The role of NFκB-dependent gene expression in regulating the growth of developing peripheral neurons

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Declarations

I, Valerie Anne Hale, hereby certify that this thesis has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree

18/11/05.

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Abstract

The principal aim of this thesis was to investigate the role of nuclear factor-kappa B (NFκB) in the developing nervous system. NFκB is a ubiquitously expressed transcription factor that plays a key role in regulating the expression of genes involved in a variety of cellular processes, including innate and adaptive immune responses, stress responses, cell survival, proliferation and differentiation. In the nervous system, NFκB plays a role in regulating neuronal survival and has been implicated in learning and memory. As the elaboration and modification of neuronal processes is thought to be an important anatomical change underlying learning and memory, a major aim of the thesis was to investigate of the potential role of NFκB signalling in the control of neuronal morphology. This was done by pharmacological and genetic interference with NFκB signalling in populations of cultured peripheral sensory and sympathetic neurons.

In sensory neurons of the nodose ganglion of newborn mice cultured with BDNF, inhibiting NFκB activation with super-repressor IκB-α, BAY 11-7082 (IκB-α phosphorylation inhibitor) or N-acetyl-Leu-Leu-norleucinal (proteosomal degradation inhibitor) or inhibiting NFκB transcriptional activity with κB decoy DNA substantially reduced neurite arbour size and complexity while having no effect on survival. This novel role of NFκB signalling in regulating neurite growth and morphology was found to be restricted to neurons cultured from mice between the ages of E18 and P1, a stage of development immediately after the phase of naturally occurring neuronal death when the processes and connections of the remaining neurons are extensively modified and refined. Monitoring NFκB dependent transcriptional activity with a GFP reporter revealed a basal level of activity that was unaffected by BDNF. NFκB was also found to be involved in promoting neurite growth from nodose neurons grown with CNTF, but not when these neurons were grown with the related cytokine LIF.

Investigating the potential role of NFκB signalling in regulating the growth of sympathetic neurites was complicated by the fact that NFκB signalling is involved in

mediating the survival-promoting effects of NGF, the neurotrophin that promotes the survival of these neurons during development. To circumvent this problem, caspase inhibitors were used to prevent the death of superior cervical ganglion neurons in which NFκB activation was prevented using either the inhibitor peptide SN50 or BAY 11-7082. These treatments resulted in significantly smaller neurite arbours seen in the presence of NGF, suggesting that NFκB also plays a role in regulating the growth and complexity of developing sympathetic neurons.

Reg-2 is a secreted signalling intermediate in CNTF-induced survival of motor neurons that activates NFκB. The above demonstration that NFκB regulates the growth, but not the survival, of newborn nodose neurons in response to CNTF raised the question of whether Reg-2 plays a role in either process in these neurons. In the final part of this thesis I began to look at the relationship between Reg-2 and CNTF in these and other peripheral neurons. Reg-2 promoted the survival of nodose but not trigeminal neurons, although its mRNA was expressed in both ganglia during a restricted period of development. *In vivo*, this expression required the transcription factor STAT-3, but not IKKα or p65, and *in vitro* CNTF or LIF, but not neurotrophins, may be able to support neuronal expression of Reg-2. These results suggest that while Reg-2 might be an intermediate for cytokine-induced neuronal survival in some neuronal populations, this is not a universal requirement, and that its expression in some neuronal populations may have another purpose.

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Contents

Table of Figures

Introduction

The vertebrate nervous system is the most complex biological system known. While there are only a few basic cells types, the diversity within these types, particularly neurons, is astounding. Each neuron interacts in a specific manner with many other neurons, as well as a number of other cell types in the nervous system and in tissues throughout the body. Study of the nervous system can be approached in many ways, from examining individual cells to whole systems, in health and disease. Alternatively insight can be gained into the function of this complex network of interactions through studying its formation.

1.1. The Development of the Vertebrate Nervous System

During the earliest stages of vertebrate development systematic expression of the genetic blueprint, in combination with environmental feedback, results in periods of intensive cell division and transformation that take an organism from a single fertilised egg to a fully formed organism. Development of the nervous system begins with a series of coordinated events known as gastrulation. The monolayered blastula becomes a more complex, three-layered structure, with different types of tissue originating from each of these layers. The innermost layer, the endoderm, later forms the epithelial structures of the respiratory and digestive systems and associated organs, while the mesoderm gives rise to connective tissues, muscles and the vascular and urogenital systems. The outer layer, the ectoderm, is the layer from which the nervous system and epidermis are derived. A series of morphogenetic movements described as neural induction first form a region called the dorsal ectoderm, which thickens to become the neural plate. The lateral edges of this plate then rise up as the neural fold. As the midline of the neural plate sinks, eventually these edges come together and fuse in a rostro-caudal direction (Smith and Schoenwolf, 1997). This forms a hollow structure called the neural tube. The cavity of this tube gives rise to the ventricular system of the central nervous system, while the neuroepithelial walls generate the central neurons and glia.

The peripheral nervous system (PNS) forms a bridge between the central nervous system (CNS) and the rest of the body, and consequently the organisms external environment. It forms principally from a layer of ectodermal cells along the border between the neural tube and the incipient epidermis called neural crest cells. Once formed these cells break free from the ectoderm and migrate along well-defined pathways through the mesoderm, where they develop into a range of cells types. Neural crest cells form the majority of the peripheral nervous system, including the autonomic, sympathetic and parasympathetic ganglia, most sensory ganglia, Schwann cells and satellite cells, as well as giving rise to chromaffin cells in the adrenal medulla, melanocytes and craniofacial connective tissues (Le Douarin et al., 2004). The remaining neurons of the PNS, those of certain cranial sensory neurons, are derived from another group of progenitor cells known as the neurogenic placodes, epithelial thickenings that occur on the rostral ectoderm of the developing embryo (D'Amico-Martel and Noden, 1983; Le Douarin, 1986; Webb and Noden, 1993). Although these two cell types have different origins, they have overlapping patterns of gene expression that reflect the commonalities in their tasks, such as delamination and migration, performed to give rise to mature, related, cell types (Mccabe et al., 2004).

1.1.1. Development of the Peripheral Nervous System

The peripheral nervous system is divided into the somatic and autonomic nervous systems, each composed of different types of neurons innervating different target fields. The neuron cell bodies are segregated into ganglia or are found in neural plexuses. These ganglia and plexuses form after the precursor cells have migrated to the appropriate locations.

1.1.1.1. Sensory Ganglia of the Peripheral Nervous System

The peripheral sensory ganglia consist of cranial sensory ganglia and the dorsal root ganglia (DRG). The seven cranial sensory ganglia are found on five of the twelve pairs of cranial nerves (Le Douarin, 1986; Weston, 1962). The trigeminal ganglion is located on cranial nerve V and its neurons innervate the mechanoreceptors, thermoreceptors and nociceptors in the face as well as providing sensory innervation

to the teeth. The geniculate ganglion, on cranial nerve VII, innervates the taste buds from the anterior two-thirds of the tongue and the vestibulo-cochlear ganglia (cranial nerve VIII), the hair cells of the organs of hearing and balance. The petrosal ganglion is located on cranial nerve IX and innervates the taste buds from the posterior third of the tongue and the jugular and nodose ganglia, which are located on cranial nerve X, innervate the pharynx, thorax and abdomen. The remaining ganglion is the superior glossopharyngeal ganglion, located on cranial nerve IX. Additionally the trigeminal mesencephalic nucleus, located in the midbrain but originating from neural crest cells, contains the cell bodies of the proprioceptive neurons innervating the muscles of mastication (Weston, 1962). The other ganglia of the sensory nervous system, the dorsal root ganglia are located adjacent to the spinal cord, on the dorsal roots of the spinal nerves. These neurons innervate the skin, muscles and joints of the limbs and trunk.

1.1.1.2. Autonomic Ganglia of the Peripheral Nervous System

The autonomic nervous system has three divisions, sympathetic, parasympathetic and enteric. The sympathetic ganglia are divided into paravertebral and prevertebral ganglia, with the paravertebral ganglia comprising a chain of ganglia lying on the ventral aspect of the vertebral column and the prevertebral ganglia being found in groups related to the major blood vessels in the abdomen. Principal paravertebral sympathetic ganglia include the superior cervical ganglion (SCG), middle cervical ganglion, stellate ganglion and sympathetic chain ganglia which innervate the salivary, lacrimal and sweat glands, as well as the vessels and hair follicles of the head and neck (SCG), heart and lungs (middle cervical and stellate ganglia) and other thoracic viscera (sympathetic chain). Prevertebral sympathetic ganglia, including the coeliac, aorticorenal and superior and inferior mesenteric ganglia, innervate organs of the digestive and urogenital tracts. Parasympathetic ganglia are found close to, or embedded in, their visceral targets and innervate the ciliary muscle, lacrimal gland, pharyngeal gland, submandibular gland, parotid gland, viscera of the thoracic and abdominal cavity and organs of the urogenital tract.

The enteric nervous system is largely autonomous from the rest of the nervous system, although its function is regulated by innervation from the sympathetic and parasympathetic nervous systems. It is composed of networks of interconnected sensory neurons, interneurons, motoneurons and astrocyte-like glia arranged in two major groups, the submucosal and myenteric plexuses, embedded in the gastrointestinal walls and provides innervation which controls the coordinated muscle movements necessary for digestion, as well as innervating the vessels and glands of the gastric mucosa.

1.1.1.3. The Origins of the Peripheral Nervous System

Sensory neurons arise from the proliferation and differentiation of progenitors derived from either the neural crest or neurogenic placodes. Neural crest cells form as a consequence of local interactions between the epidermis and neural plate and segregate around the time that the neural tube closes (Selleck and Bronner-Fraser, 1995), while the neurogenic placodes are induced by mesoderm, neuroectoderm and endoderm depending on the placode (Baker et al., 1999; Stark et al., 1997)reviewed in (Baker and Bronner-Fraser, 2001). Detailed mapping experiments involving the transplantation of neural crest- and placode-derived cells have determined the origins of cranial sensory ganglia in avians. The ventrolateral part of the trigeminal ganglia and the vestibulo-cochlear, geniculate, petrosal and nodose ganglia are derived from neurogenic placodes, while the dorsomedial trigeminal ganglion, trigeminal mesencephalic nucleus, jugular ganglion and dorsal root ganglion are derived from cranial neural crest (D'Amico-Martel, 1982; D'Amico-Martel and Noden, 1983; Le Douarin, 1986; Le Douarin et al., 2004; Noden, 1978; Webb and Noden, 1993). In the autonomic nervous system, both neurons and glia of the parasympathetic ganglia arise from mesencephalic neural crest cells, while the enteric nervous system is formed from the vagal and lumbosacral neural crest (Epstein et al., 1994; Le Douarin et al., 2004; Serbedzija et al., 1991). Sympathetic neurons are generated from the entire length of the trunk neural crest (Le Douarin et al., 2004). Additionally, all Schwann and satellite cells associated with all sensory and autonomic ganglia are derived solely from the neural crest (D'Amico-Martel and Noden, 1983). Grafting experiments have also revealed that both presumptive placodal ectoderm and pre-

migratory neural crest cells are not specified to differentiate into particular sensory neurons, but are multipotent, their phenotype being influenced by external factors along their migratory path and at their destination site (Vogel and Davies, 1993; Le Douarin et al., 2004).

1.2. Regulation of cell survival and neuronal number in the developing peripheral nervous system

1.2.1. The neurotrophic hypothesis...

During the development of the nervous system, neurons are produced in quantities exceeding the total number of cells required to establish a functional nervous system. Final cell number is determined during a subsequent phase of apoptotic cell death when over half of the developing neurons die (Burek and Oppenheim, 1996). The survival of each cell is determined by the limited availability of neurotrophic factors, usually derived from the innervation target. This pattern of excess cell production followed by cell death is thought to tailor the number of neurons to the size of the target tissue and to eliminate any neurons whose cell bodies are wrongly located or that have formed inappropriate connections. It has been termed the "neurotrophic theory" (Davies, 1994b).

The neurotrophic hypothesis was formulated following the study of the classical neurotrophic factor, nerve growth factor (NGF). Sensory and sympathetic neuronal populations that are NGF-dependent *in vitro* were found to be eliminated *in vivo* upon administration of anti-NGF or anti-NGF-receptor antibodies at the time of target field innervation (Henderson et al., 1994; Johnson, Jr. et al., 1989; Johnson, Jr. and Yip, 1985; Levi-Montalcini, 1987; Levi-Montalcini and Angeletti, 1968; Pearson et al., 1983; Yip et al., 1984). Exogenous NGF administered at this time also rescued additional neurons, resulting in an increased neuronal number in the relevant ganglia (Henderson et al., 1994; Yip et al., 1984). More recently use of transgenic technologies have confirmed these effects, with null mutations of the genes coding for either NGF or its receptor tyrosine kinase, TrkA, resulting in the loss of the same populations of peripheral neurons (Crowley et al., 1994; Henderson et al., 1994;

Smeyne et al., 1994) and overexpression of neurotrophins, such as NGF, in the target fields such as the skin resulting in increased numbers of sensory neurons surviving (Albers et al., 1994; Davis et al., 1997). Additional support for this hypothesis has been gained by studying the site, timing and level of expression of NGF. Initiation of NGF synthesis in the relevant peripheral target fields occurs as target field innervation begins (Davies et al., 1987; Korsching and Thoenen, 1988) and increased cell death is seen when specific target fields are removed during development (Caldero et al., 1998).

1.2.2. ...and beyond

Since the discovery of NGF several other neurotrophic factors have been described. Studies of the effects of these proteins on neurons, both *in vitro* and *in vivo*, and of the consequences of introducing null mutations in these proteins and their receptors, have confirmed similar patterns to that of NGF (Baloh et al., 2000; Davies, 2003; Huang and Reichardt, 2001; Turnley and Bartlett, 2000). Recent evidence has also indicated, however, that the situation is more complex than initially described in the neurotrophic hypothesis, and that different types of neurons require trophic support from sources other than their target fields at points both before and after target field innervation (Davies, 2003). As well as neurotrophic factors derived from target fields, neurons may also need trophic support before target field innervation. This can be derived from developing cells on the pathway through which the neurons are extending processes, in an autocrine/paracrine manner from neighbouring neurons or after target field innervation (Acheson et al., 1995; Caldero et al., 1998; Maina et al., 1998; Riethmacher et al., 1997; Robinson et al., 1996b; Wright et al., 1992). It has also become clear that most populations of neurons have trophic requirements that change depending on their stage of development and which often overlap (Davies, 1994a; Davies, 1997; Ernfors et al., 1994b; Ernfors et al., 1994a; Francis et al., 1999; Hohn et al., 1990; Jones et al., 1994; Wyatt et al., 1997; Zhou and Rush, 1995). Additionally, exposure of neurons to many trophic factors have consequences over and above those of neuronal survival including influencing precursor proliferation and differentiation, process outgrowth, neurotransmitter and neuropeptide phenotype,

and synaptic form and function throughout life (Bibel and Barde, 2000; Davies, 2000a; Davies, 2000b; English, 2003; Maina et al., 1998; Thoenen, 2000).

1.2.3. The effects of growth factors on the survival of the developing sensory and sympathetic nervous systems

The studies in this thesis are performed on sensory neurons of the nodose and trigeminal ganglia and sympathetic neurons of the superior cervical ganglion. In this section I will therefore discuss what is known about the trophic requirements of these ganglia. Where the responses of avian and mammalian neurons diverge I will consider only the mammalian responses, as it is murine neurons that are used in my studies.

1.2.3.1. The sensory ganglia

Neurons of the trigeminal ganglia have been shown to depend on a number of different neurotrophic factors throughout their development. Neurons generated during the earliest stages of trigeminal ganglia formation are initially dependent on brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) for their survival. By E12, however, very few trigeminal neurons are BDNF-dependent, with NGF being their preferred survival factor (Buchman and Davies, 1993; Paul and Davies, 1995). There are two reasons for this change in neurotrophic factor dependence. Firstly, neurons generated in the trigeminal ganglia after E12 respond immediately to NGF and never possess the ability of the earlier generated neurons to respond to BDNF or NT-3 (Enokido et al., 1999; Huang et al., 1999). Additionally, early neurons that respond to BDNF and NT-3 alter their trophic responses as they develop (Enokido et al., 1999). The change in responsiveness of trigeminal neurons from BDNF and NT-3 to NGF is correlated with a change in receptor expression, as well as in neurotrophin mRNA expression in the peripheral target fields (Arumae et al., 1993; Buchman and Davies, 1993; Davies et al., 1987; Ninkina et al., 1996; Wyatt and Davies, 1993). Analysis of the timing of excessive cell death in the trigeminal ganglia of mice with null mutations of the receptor genes TrkA and TrkB (NGF and BDNF receptors respectively) suggests that these changes are relevant *in vivo*. Increased apoptosis in the trigeminal ganglia is seen at an earlier stage in $trkB^{-/-}$

or nt-3$^{-/-}$ embryos than in trkA$^{-/-}$ embryos (Pinon et al., 1996; Wilkinson et al., 1996). BDNF and NT-3 are expressed in the tissues through which neuronal processes extend suggesting that they provide intermediate trophic support before the neuronal extension reaches the target fields (Buchman and Davies, 1993; Davies, 1997; Pinon et al., 1996; Wilkinson et al., 1996).

The change in the trophic requirements of trigeminal neurons from BDNF and NT-3 to NGF before E12 is not the only change in trophic factor responsiveness that occurs during development. Whilst *in vitro* NGF retains the ability to promote neuronal survival past birth, its dose response curve shifts significantly so that 10-fold higher concentrations of NGF are required to promote a maximal effect (Buchman and Davies, 1993). During this same period of development, between E16 and E18, murine trigeminal neurons acquire the ability to respond to macrophage-stimulating protein (MSP), which subsequently promotes *in vitro* survival at least as well as NGF (Forgie et al., 2003). Around half of all trigeminal neurons acquire the ability to respond to cytokines such as ciliary neurotrophic factor (CNTF) at the same age. (Horton et al., 1998). Members of the glial-derived neurotrophic family (GDNF) family of trophic factors are also involved in supporting trigeminal neuron survival during development, as neurturin$^{-/-}$ mice show a significant loss of neurons in the trigeminal ganglia (Heuckeroth et al., 1999).

Nodose neurons survive in the absence of trophic support when they are first generated. Dependence on neurotrophins for survival is acquired later in development, at the same time as the expression of neurotrophin receptors (Robinson et al., 1996a; Vogel and Davies, 1991). The *in vitro* survival of nodose neurons is subsequently supported by the neurotrophins BDNF, NT-3 and NT-4 (Buj-Bello et al., 1994; Davies et al., 1986; Davies et al., 1993; Lindsay et al., 1985; Vogel and Davies, 1991). That these *in vitro* effects are relevant *in vivo* has been well established, as increased neuronal death during embryonic development can be caused by deletion of the genes coding for BDNF, NT-3 and NT4, or their receptors TrkB and TrkC, and increased neuronal number is found in mice overexpressing BDNF in the skin (Conover et al., 1995; ElShamy and Ernfors, 1997; Erickson et al., 1996; Farinas et al., 1994; Klein et al., 1993; LeMaster et al., 1999; Liu et al., 1995).

Recently a small population of early nodose neurons (E12-E13) were also found to survive transiently in the presence of NGF *in vitro* and small decreases in neuronal number have been detected in the $NGF^{-/-}$ mouse (Forgie et al., 2000). As with the trigeminal ganglia, nodose neurons also respond to a range of other neurotrophic factors. Early in development, neurotrophic cytokines, including CNTF, leukaemia inhibitory factor (LIF), oncostatin M (OsM) and cardiotrophin-1 (CT-1), support the survival of a subpopulation of nodose neurons (Horton et al., 1998). As development continues an increasing proportion of nodose neurons respond to these factors, so that by birth most neurotrophic cytokines promote survival as effectively as BDNF (Horton et al., 1998). Neurons of the nodose ganglia also respond to neurturin during development and decreases in neuronal number have been found in the nodose ganglia in $GDNF^{-/-}$ mice (Kotzbauer et al., 1996).

1.2.3.2. The sympathetic ganglia

Like sensory neurons, neurons of the sympathetic ganglia show robust survival responses to a range of trophic factors *in vitro* and the responses to these factors change depending on the stage of neuronal development. New SCG neurons are generated from a proliferating precursor population until E14/15. Artemin and hepatocyte growth factor (HGF) initially promote precursor proliferation and survival respectively, while artemin alone promotes the survival of newly generated neurons in mice and rats, a response that is lost by E16 (Andres et al., 2001; Baloh et al., 1998; Maina et al., 1998). MSP also promotes the survival of a subset of neurons at E14, but by E17 has no trophic effect (Forgie et al., 2003). Murine sympathetic neurons start to respond to NGF at E14, so that by E18, shortly before birth, the majority of these neurons depend on NGF for their survival (Wyatt and Davies, 1995). Additionally, between E16 and E18, a smaller proportion of sympathetic neurons become NT-3 dependent *in vivo* (Birren et al., 1993; Wyatt et al., 1997).

Further changes in the trophic factor responses of SCG neurons occur after birth. SCG neurons retain their ability to use NGF as a survival factor, however the proportion of neurons that also survive in the presence of NT-3 increases so that postnatal sympathetic neurons are both NGF and NT-3 dependent (Belliveau et al.,

1997; Davies et al., 1995; Davies, 1997; Fagan et al., 1996; Francis et al., 1999; Levi-Montalcini, 1987; Wyatt et al., 1997; Zhou and Rush, 1995). A phase of neurotrophic response to artemin also occurs after birth, starting at P12 and continuing into adulthood. This effect begins at the same time as over half of SCG neurons are acquiring the ability to survive in the absence of trophic support. Artemin, at this stage, promotes the survival of an additional 25% of neurons *in vitro* (Andres et al., 2001). During the postnatal period a significant loss of SCG neurons is seen in mice lacking GFRα3, the ligand binding portion of the artemin receptor, confirming the *in vivo* relevance of this change (Nishino et al., 1999). Postnatal sympathetic neurons of the SCG also acquire survival responses to CNTF, LIF (Kotzbauer et al., 1994) and HGF. HGF has been found to promote the survival of late postnatal sympathetic neurons (P20), an effect that continues into adulthood when these neurons have lost all NGF-responsiveness (Thompson et al., 2004).

1.3. Regulation of axon and dendrite formation and the establishment of innervation in the peripheral nervous system

To establish a functional nervous system, neurons must not only develop in the correct number and location, but they must also form functional connections with the appropriate targets to allow participation in local and global neuronal circuitries. This occurs through a number of mechanisms regulating the number, length, branching and direction of growth of cell processes, as well as synaptogenesis. Intrinsic developmental programs regulate some of the variations between neuronal types, however a complex variety of extracellular signals are also essential to establishing functional connections (Goldberg, 2004). These extracellular stimuli include soluble, cell surface and extracellular matrix proteins, a large number of which are also implicated in either cell proliferation, survival or migration (de Vellis and Carpenter, 1999). In line with the focus of the thesis, in this section I will introduce the basis of neurite outgrowth and move on to discuss the roles that neurotrophic factors play in this process. The effects of molecules such as guidance molecules and extracellular matrix components will not be discussed in detail.

The sequence of events occurring during neuritic growth has been well characterised for neurons in culture. First, a number of neuritic processes are extended and retracted by postmitotic neurons, with each process being both morphologically and immunologically indistinguishable from any other process. All early processes express both actin- and microtubule-associated proteins such as cofilin, tau and MAP2 (Keith and Wilson, 2001). Initiation of process growth, however, is dependent on the actin cytoskeleton, whereas the subsequent process elongation is microtubule-dependent (Smith, 1994). In time, one neurite extends further than other processes and develops axonal characteristics, becoming a non-tapering process that shows increased expression of tau, but loses MAP2 expression. Subsequently, other processes develop into dendrites, shorter tapering processes characterised by robust expression of MAP2. Both tau and MAP2 appear essential to the specification of neurite phenotype, and interfering with their expression is capable of blocking axonal or dendritic specification respectively and causing retraction of previously developed neurites (Heidemann, 1996; Mandell and Banker, 1995; Schoenfeld and Obar, 1994; Tucker, 1990).

In vivo the selection of the neurite that will become the axon is thought to be a stochastic process, with the direction of growth determined by local environmental cues. Each developing neuritic process is specialised at the tip into a structure called a growth cone, which differs from the shaft of an axon or dendrite in that its structure is formed from a highly dynamic actin-based cytoskeleton. Within the growth cone two different motile structures, filopodia and lamellipodia, interact with the environment outside the cell, interpreting both pathfinding and outgrowth cues. Lamellipodia contain branched networks of actin filaments that are principally involved in elongation, whereas filopodia, mediating axonal guidance, are formed from bundled actin filaments that interact with the microtubules from the shafts to reinforce directional choices (Letourneau, 1996). Several different types of external input can activate pathways that mediate outgrowth and pathfinding, from proteins embedded in the structure of the extracellular matrix, to neurotrophic factors and guidance molecules, neurotransmitters and electrical activation (Keith and Wilson, 2001; Tessier-Lavigne and Goodman, 1996; Wong and Ghosh, 2002). These inputs

ultimately alter the size and shape of neurons by regulating the assembly, stabilisation and disassembly of the neuronal cytoskeleton (Gallo and Letourneau, 2000; Miller and Kaplan, 2003). As with cell survival, factors influencing neurite outgrowth can be found local to the cell body, throughout the route along which the neurites are extending or in the target field (Miller and Kaplan, 2003).

1.3.1. The effects of growth factors on neurite outgrowth in the developing peripheral nervous system

Many different proteins have been implicated in the promotion of differing types of neurite outgrowth in the developing peripheral nervous system. The most studied type of neurite outgrowth in the nervous system is axonal outgrowth (Keith and Wilson, 2001). The majority of this section will therefore describe the role of neurotrophic factors in promoting axonal growth, but wherever possible I have also discussed how this compares to dendritic growth in the same, or related, neuronal populations.

As with survival, the neurotrophins are one of the best studied families of proteins with respect to neurite outgrowth. Abnormalities in axonal projections of the vestibular and cochlear ganglia are found in mice lacking BDNF, NT-3, trkB and trkC and in sympathetic axons of $trkA^{-/-}$ mice (Ernfors et al., 1995; Fagan et al., 1996; Postigo et al., 2002; Schimmang et al., 1995). The essential role of neurotrophins in neuronal survival in many populations complicated the early study of their role in neurite outgrowth (Davies et al., 1987; Lumsden and Davies, 1983; Scott and Davies, 1993). Recently these problems have been overcome by using new compounds or transgenic mice to block apoptosis and allow neurons to survive both *in vivo* or *in vitro* in the absence of trophic support (Deckwerth et al., 1996; Lentz et al., 1999; Markus et al., 2002b; Patel et al., 2000; Werth et al., 2000; White et al., 1998). For example, Patel et al (2000) crossed TrkA or NGF null mice with mice lacking the pro-apoptotic protein Bax, a manipulation that rescues peripheral sensory neurons lost in the TrkA or NGF single knockout mice. In the DRG of these mice the central axon projections extend normally into the dorsal roots and spinal cord, but the peripheral axonal projections are absent. This may be a consequence of a genuine role of NGF in regulating neurite outgrowth of DRG neurons or alternatively due to a

role of NGF in the maintenance of the peripheral projection. Further evidence supports the conclusion that NGF has a role to play in promoting neurite outgrowth in these neurons. *In vitro* studies using E11-E13 $Bax^{-/-}$ DRG neurons find that in the absence of additional trophic factors neurons extend a single short projection, but become bipolar with robust neurite extension in the presence of NGF, NT-3 or BDNF (Lentz et al., 1999). In organotypic slice cultures the role of neurotrophins in axonal growth and guidance has been further confirmed, as insertion of ectopically-placed beads coated in NGF, BDNF, NT-3 or NT-4/5 caused changes in axon targeting and injection of antibodies against NGF, BDNF or NT-3 caused dramatic decreases in axonal extension in DRG neurons after E10.75 (Tucker et al., 2001).

Despite the common role of NGF, BDNF and NT-3 in neurite outgrowth described above, individual neurotrophins differ in their precise effect in shaping the neuronal arbour. Lentz et al (1999) showed that $Bax^{-/-}$ DRG neurons growth with NGF or NT-3 elaborated neurites with very differently morphologies. In the presence of NGF neurons extended long neurites with few branches until the terminal third of the neurite, whereas neurons grown with NT-3 had much shorter neurites that showed earlier and more extensive branching. BDNF initiated morphologies similar to those with NT-3. Such a difference is confirmed by the changes in elongation and branching that occur when wholemount explants of trigeminal ganglia connected to their target fields are exposed to NGF or NT-3, or the changes in innervation that occur in the trigeminal target when either NGF or NT-3 is overexpressed in the target field *in vivo* (Davis et al., 1997; Rice et al., 1998; Ulupinar et al., 2000). Both NGF and NT-3 also support neurite outgrowth by sympathetic neurons *in vitro* and again their roles appear different (Campenot, 1982). While $NGF^{-/-}$ mice show relatively normal axon elongation, but defects in final target innervation, $NT\text{-}3^{-/-}$ mice exhibit decreased axonal extension long before the target field is reached (Glebova and Ginty, 2004; Kuruvilla et al., 2004). This suggests that NT-3 is an intermediate target-derived factor, promoting neurite extension of sympathetic neurons before neurons are exposed to NGF. Adding further complexity to our understanding of neurotrophin-mediated regulation of sympathetic neurite outgrowth, Belliveau et al (1997) also found that NT-3, as well as NGF, promotes outgrowth via TrkA, rather

than TrkC its preferred receptor. Activation of intracellular signalling pathways was, however, temporally different. Additionally BDNF, acting via p75 in TrkB-negative sympathetic and DRG neurons, appears to antagonise NGF-induced neurite outgrowth (Kohn et al., 1999; Paves and Saarma, 1997).

Neurotrophins have also been implicated in dendritic formation, both *in vitro* and *in vivo*. Injection of excess NGF *in vivo* causes increased dendritic growth in sympathetic neurons (Ruit et al., 1990; Ruit and Snider, 1991; Snider, 1988). This effect only occurs *in vitro* in the presence of non-neuronal cells or with the co-operation of additional growth factors such as bone morphogenetic protein-7 (BMP-7), a soluble protein that is found associated with the basement membrane (Bruckenstein and Higgins, 1988; Lein et al., 1995; Lein et al., 1996; Tropea et al., 1988). NGF can also enhance activity-dependent dendritic outgrowth in sympathetic neurons (Vaillant et al., 2002). Evidence of a role for BDNF and NT-3 in dendritic growth comes principally from studies of specific populations of central, rather than peripheral neurons. In most cases these neurotrophins promote dendritic outgrowth, however in some cortical neurons it has been reported that the actions of BDNF and NT-3 can be mutually antagonistic (Baker et al., 1998; Gascon et al., 2005; Horch et al., 1999; Horch and Katz, 2002; Jin et al., 2003; Kohara et al., 2003; Labelle and Leclerc, 2000; McAllister et al., 1995; McAllister et al., 1996; McAllister et al., 1997; Sang and Tan, 2003; Wirth et al., 2003; Xu et al., 2000).

Neurotrophins are not the only family of neurotrophic factors implicated in neurite outgrowth and the establishment of innervation in the PNS. HGF can facilitate NGF-induced neurite outgrowth in sensory neurons (Maina et al., 1997). Outgrowth of sympathetic neurites is significantly impaired in embryos lacking Ret, a common receptor subunit for GDNF family ligands, while survival is unaffected (Enomoto et al., 2001). Vascular-derived artemin is also thought to promote axonal extension en route to the target field, as it can promote outgrowth and attract neurites *in vitro* and as defects similar to those in the $Ret^{-/-}$ embryos are seen in artemin and GFRα3 knockout mice (Enomoto et al., 2001; Honma et al., 2002). GDNF itself supports axonal growth in the parasympathetic neurons of the ciliary ganglion both *in vitro* and *in vivo* (Hashino et al., 2001). Neurturin has also been found to be important in

the establishment of innervation of certain types of sympathetic and parasympathetic neurons (Hiltunen and Airaksinen, 2004; Laurikainen et al., 2000; Rossi et al., 1999; Rossi et al., 2000; Rossi and Airaksinen, 2002).

Some evidence implicates neuropoietic cytokines in influencing neurite outgrowth, although the majority of these studies are again in the CNS. Neurite outgrowth is promoted by CNTF in retinal gangion cells and LIF in spinal cholinergic and auditory neurons (Gillespie et al., 2001; Goldberg et al., 2002; Richards et al., 1992). CNTF and LIF are also associated with plasticity-related changes, such as sprouting, in primary sensory neurons and at the neuromuscular junction (English, 2003; Gurney et al., 1992; Ramer et al., 1999; Tarabal et al., 1996; Thompson and Majithia, 1998). In contrast to this, however, both LIF and CNTF have been found to inhibit dendritic outgrowth and cause dendritic retraction in sympathetic neurons where growth is promoted by BMP-7 (Guo et al., 1997; Guo et al., 1999).

1.4. The biology of neurotrophic factors

There are many proteins that have been described as having the ability to promote the survival of neurons, both in vitro and in vivo, and which can thus be described as "neurotrophic factors". Some of these factors, such as NGF, were originally found in this context (Levi-Montalcini and Angeletti, 1968), whereas others, such as the neuropoietic cytokine LIF, were initially described in the context of different systems, and subsequently found to be active in the nervous system. The principal families of neurotrophic factors are: the neurotrophins (NGF, BDNF, NT-3 and NT-4), the GDNF family (GDNF, neurturin, artemin and persephin), the neuropoietic cytokines (CNTF, LIF, OSM, CT-1, IL-6, CLC and neuropoietin) and two related factors HGF and MSP. All of these factors have been found to promote neuronal survival, however it is important to remember that none of these proteins act exclusively in this manner. During development, neurotrophic factors have also been found to promote the proliferation and differentiation of both neuronal and non-neuronal precursors, to regulate neurite outgrowth and the establishment of innervation, and to influence the eventual neurotransmitter and neuropeptide phenotype of developing neurons (Airaksinen and Saarma, 2002; Davies, 2000a;

Maina and Klein, 1999; Turnley and Bartlett, 2000). Neurotrophic factors have also been found to be important throughout life, affecting neuronal outgrowth and branching, synaptic plasticity and the stabilisation of synaptic contacts, as well as being involved in regulating the consequences of injury and disease (Binder and Scharfman, 2004; Boyd and Gordon, 2003; English, 2003; Kojima et al., 2002; Mendell et al., 2001; Villoslada and Genain, 2004). In this section I will further describe the families of neurotrophic factors that I use in my investigations, that is the neurotrophin family and the neuropoietic cytokines of the IL-6 family, and their roles in the development of the nervous system.

1.4.1. Neurotrophins and their receptors

The neurotrophin family of proteins has 4 members in reptiles, amphibians, birds and mammals: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) neurotrophin-3 (NT-3), neurotrophin 4/5 (NT-4/5), and two additional members in fish: neurotrophin 6 (NT-6) and neurotrophin-7 (NT-7). These proteins are both structurally and functionally secreted proteins that signal through two classes of receptor. These receptors are the high-affinity tyrosine kinase receptors of the Trk family (TrkA, TrkB, and TrkC) and a common lower-affinity receptor, p75. Generally neurotrophins are important regulators of neuronal survival, axonal growth and synaptogenesis (Huang and Reichardt, 2001).

1.4.1.1. Nerve growth factor

Nerve growth factor was the first neurotrophin to be isolated, in 1959, when it was purified from snake venom for its ability to promote neurite outgrowth and subsequently found to be expressed in high levels in the submandibular salivary gland of the adult mouse (Cohen, 1960; Cohen, 1959). The cloning of both the avian and mammalian gene in several species has since shown that NGF is a highly conserved protein (Ebendal et al., 1986; Meier et al., 1986; Ullrich et al., 1983). The murine NGF gene produces a 307 amino acid precursor protein, which is cleaved to give a 118aa mature protein containing a cysteine knot motif and two antiparallel β strands (Berger and Shooter, 1977; Edwards et al., 1988; Scott et al., 1983) and

which forms a forms a homodimer to produce a biologically active protein (McDonald et al., 1991).

NGF is expressed in several regions of the CNS, including the hippocampus, olfactory bulb and neocortex, regions associated with innervation from cholinergic neurons of the basal forebrain, as well as in the septum, diagonal band of Broca and the nucleus basalis of Meynert, where these neurons originate (Korsching et al., 1985). Outside the central nervous system, NGF can also be detected in the target tissues of neurons known to respond to NGF, including the heart, submandibular gland, and the developing mouse whisker pads and the skin in general, where it is principally produced by epithelial cells (Davies et al., 1987; Heumann et al., 1984; Korsching et al., 1985; Korsching and Thoenen, 1983; Shelton and Reichardt, 1984).

In vitro and *in vivo* studies show that NGF is a survival factor for developing sympathetic neurons, some sensory neurons and basal forebrain cholinergic neurons (Chun and Patterson, 1977; Davies and Lindsay, 1985; Hamburger et al., 1981; Hartikka and Hefti, 1988; Levi-Montalcini, 1987). Studies of the role of NGF in the developing nervous system *in vivo* have been particularly advanced by the generation of the NGF null mutant mouse, a mutation that is lethal soon after birth. $NGF^{-/-}$ mice are born with almost no sympathetic neurons, and show losses of trigeminal neurons. (Crowley et al., 1994). In the DRG, only a subpopulation of neurons, small diameter neurons thought to mediate nociception, are affected (Crowley et al., 1994). No obvious deficits on central neuronal populations have been found, suggesting that NGF alone is not an essential survival factor in the developing central nervous system. In terms of establishment of innervation, NGF has also been found be involved in neurite outgrowth in trigeminal, sympathetic and DRG neurons (Davis et al., 1997; Glebova and Ginty, 2004; Kohn et al., 1999; Kuruvilla et al., 2004; Lentz et al., 1999; Patel et al., 2000; Paves and Saarma, 1997; Snider, 1988; Tucker et al., 2001).

Recently a new aspect of neurotrophin signalling has been described. The precursor protein for NGF, pro-NGF, has traditionally thought to be inactive, however Lee et al (2001) found that pro-NGF can be released into the extracellular milieu. Here it can

be cleaved by plasmin or matrix metalloproteinases, but additionally pro-NGF can bind to the p75 receptor with high affinity, forming a receptor complex with sortilin that activates pro-apoptotic signals (Lee et al., 2001; Nykjaer et al., 2004). ProNGF has been shown to induce apoptosis after CNS-injury (Harrington et al., 2004).

1.4.1.2. Brain-derived neurotrophic factor

Brain-derived neurotrophic factor was first purified from adult porcine brain and was found to be a survival factor for early embryonic chick dorsal root ganglion neurons (Barde et al., 1982). It is a 12.3kDa basic protein that has subsequently been cloned in several species including human, mouse and rat (Hofer et al., 1990; Jones and Reichardt, 1990; Maisonpierre et al., 1991). Like NGF, BDNF is translated as a precursor protein that is cleaved to produce an 119aa mature protein. BDNF shares approximately 50% of its sequence with NGF, including the 6 cysteine residues involved in forming the cysteine knot structure, and also forms a homodimer to produce its biologically active species (Radziejewski et al., 1992).

Low levels of BDNF expression in the early embryonic CNS become higher and more widespread as the CNS matures, with robust expression detected in the cortex, hippocampus and cerebellum of adult mice, as well as lower levels detectable in the striatum, olfactory bulb, midbrain, hindbrain and spinal cord (Hofer et al., 1990; Leibrock et al., 1989). In the PNS, BDNF expression is found in neurons of the dorsal root, jugular and trigeminal ganglia (Robinson et al., 1996a; Schecterson and Bothwell, 1992). BDNF expression is also found in the target tissues of BDNF-responsive neurons, including the heart, skin, muscle, and lung (Buchman and Davies, 1993; Maisonpierre et al., 1990; Maisonpierre et al., 1991; Schecterson and Bothwell, 1992).

During development of the PNS, BDNF promotes the survival of embryonic neural crest-derived neurons of the trigeminal mesencephalic nucleus and the dorsal root ganglia and the placode-derived neurons of the trigeminal ganglia, geniculate, petrosal, vestibular and nodose ganglia (Barde et al., 1987; Davies et al., 1986; Hofer and Barde, 1988; Lindsay et al., 1985). In the CNS, BDNF enhances the survival and

differentiation of a range of neurons including basal forebrain cholinergic neurons, mesencephalic dopaminergic neurons, motoneurons, hippocampal neurons, cerebellar granule cells and retinal ganglion neurons (Alderson et al., 1990; Hyman et al., 1991; Johnson et al., 1986; Knusel et al., 1992; Lindholm et al., 1993; Oppenheim et al., 1992). As with pro-NGF, pro-BDNF can activate apoptotic signalling through p75 (Lee et al., 2001). Mice with a homozygous null mutation of the BDNF gene exhibit peripheral nervous system defects including substantial loss of trigeminal, geniculate, vestibular, petrosal and nodose neurons, but no overt defects of the central nervous system (Ernfors et al., 1994a; Jones et al., 1994). Defects are therefore principally of sensory neurons, with sympathetic, dopaminergic or motoneurons being unaffected (Ernfors et al., 1994a; Jones et al., 1994). Like the NGF null mutation, this mutation is lethal shortly after birth.

In the peripheral nervous system, BDNF is involved in neurite outgrowth in vestibular, DRG, sympathetic and nodose neurons (Fritzsch et al., 1997; Kohn et al., 1999; Lentz et al., 1999; Paves and Saarma, 1997; Postigo et al., 2002; Schimmang et al., 1995; Tucker et al., 2001). In the central nervous system, BDNF and TrkB signalling are necessary for the growth and maintenance of hippocampal and cortical dendrites (Horch et al., 1999; Horch and Katz, 2002; Jin et al., 2003; Kohara et al., 2003; Labelle and Leclerc, 2000; McAllister et al., 1997; Sang and Tan, 2003; Wirth et al., 2003; Xu et al., 2000) with local overexpression of BDNF in cortical neurons causing only local increases in neurite outgrowth (Horch and Katz, 2002). The source of the neurotrophin may change how it affects neurite outgrowth, as it has been shown that distal BDNF can promote dendrite formation in venous retinal ganglion cells, but that applied to the cell body it decreases dendritic branching (Lom et al., 2002).

1.4.1.3. Neurotrophin-3

NT-3 is another member of the neurotrophin family important in the development of the nervous system. It was discovered using oligonucleotide primers selected for the conserved regions of the NGF and BDNF genes and its genomic sequence has been cloned in several mammalian species including humans, mice and rats (Hohn et al.,

1990; Jones and Reichardt, 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990). Again, NT-3 is produced as a 258-aa precursor that is subsequently cleaved to produce 119aa mature protein that shares 50-60% homology with NGF and BDNF (Hohn et al., 1990).

In the peripheral nervous system NT-3 is expressed principally in the epithelium of the whisker follicles, that is the trigeminal target field, with expression peaking early in embryonic development and decreasing thereafter (Buchman and Davies, 1993; Hallbook et al., 1993; Maisonpierre et al., 1990; Rosenthal et al., 1990). Some expression is detected in adulthood, in a range of tissues including muscle, heart, lung, liver, intestine and spleen, suggesting that NT-3 may act as a target derived trophic factor for both sensory and sympathetic neurons (Henderson et al., 1993; Hohn et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990). In the central nervous system, NT-3 is also highly expressed during development, with levels falling dramatically as these regions mature (Maisonpierre et al., 1991). In adults, expression can be detected principally in the hippocampus, cerebellum and medulla oblongata (Hohn et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990).

In vitro, subpopulations of neurons from the nodose, cochlear and dorsal root ganglia as well as the proprioceptive neurons of the trigeminal mesencephalic nucleus respond to NT-3 and a transient survival response of neural crest-derived sensory neurons can also be detected (Ernfors et al., 1990; Hohn et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990; Buchman and Davies, 1993; Buj-Bello et al., 1994; Farinas et al., 1994; Farinas et al., 1996; Ockel et al., 1996; Wilkinson et al., 1996). NT-3 also induces the survival, differentiation and proliferation of sensory neuronal precursors (ElShamy et al., 1996; ElShamy and Ernfors, 1996; Kalcheim et al., 1992; Memberg and Hall, 1995; Pinco et al., 1993). In the CNS, NT-3 is a survival factor for cultured embryonic noradrenergic neurons and motoneurons (Friedman et al., 1993; Henderson et al., 1993) and also promotes the proliferation and survival of oligodendrocyte precursors *in vitro* and *in vivo* (Barres et al., 1994). Mice lacking the NT-3 gene exhibit significant cell loss in all peripheral sympathetic and sensory neuron populations including the SCG, DRG, trigeminal and nodose ganglia, thus confirming the physiological relevance of the *in vitro* survival assays (ElShamy et

al., 1996; ElShamy and Ernfors, 1996; Ernfors et al., 1994b; Farinas et al., 1994; Farinas et al., 1996; Francis et al., 1999; Tessarollo et al., 1994; Wyatt et al., 1997). In the peripheral nervous system, NT-3 also plays a role in the establishment of innervation by vestibular, DRG, trigeminal and sympathetic neurons (Postigo et al., 2002) (Belliveau et al., 1997; Kuruvilla et al., 2004; Lentz et al., 1999; Rice et al., 1998; Schimmang et al., 1995; Tucker et al., 2001).

1.4.1.4. Other neurotrophins

Neurotrophin-4/5 was the fourth member of the neurotrophin family to be isolated. It was originally cloned from Xenopus laevis and named neurotrophin-4 by Hallbook et al. (1991). Later human and rat genes were cloned and designated NT-5 (Berkemeier et al., 1991; Ip et al., 1992) however it is now believed that the Xenopus, rat and human genes are the same therefore they are referred to as NT-4/5. Mature NT-4/5 is a 123aa protein bearing all 6 conserved cysteine residues and having approximately 50% homology with NGF, BDNF and NT-3 (Ip et al., 1992).

NT-4/5 is expressed in the developing whisker pad and limb buds, thus indicating a role in the development of trigeminal neurons and spinal motoneurons (Henderson et al., 1993; Ibanez et al., 1993). NT-4/5 is also expressed in several other embryonic and adult tissues, including heart, muscle, lung, kidney, thymus, ovary and testis (Berkemeier et al., 1991). In the CNS, NT-4/5 is expressed in the cortex, olfactory bulb, hippocampus, hypothalamus, cerebellum, pons and medulla (Timmusk et al., 1993).

In the peripheral nervous system, NT-4/5 supports the survival of embryonic trigeminal and jugular neurons *in vitro,* at ages when early target innervation is occurring, and nodose neurons during the phase of naturally occurring cell death (Davies et al., 1993). At high concentrations NT-4/5 can also support the survival of sympathetic neurons (Berkemeier et al., 1991; Hallbook et al., 1991). Unlike BDNF, NGF and NT-3, NT-4/5 does not appear to be well conserved between mammals and birds, as the effects of NT-4/5 on chick neurons are very limited (Davies et al., 1993; Ibanez et al., 1993). In the CNS, NT-4/5 promotes the survival of embryonic spinal

motoneurons, cholinergic basal forebrain neurons and noradrenergic locus coeruleus neurons (Friedman et al., 1993; Funakoshi et al., 1995). Mice lacking the NT-4/5 gene show losses of sensory neurons of the nodose, petrosal and geniculate ganglia, while sympathetic SCG neurons, facial motoneurons and midbrain dopaminergic neurons are unaffected (Conover et al., 1995; Erickson et al., 1996; Liu et al., 1995). NT-4/5 is also implicated in neurite outgrowth in adult nodose neurons (Wiklund and Ekstrom, 2000).

Neurotrophin-6 was cloned from Xiphophorus maculatus and is a 146aa mature protein containing the 6 cysteine residues characteristic of the neurotrophin family (Gotz et al., 1994). It differs from other family members by a 22aa insertion between the second and third cysteine, containing a heparin domain. Additionally it does not appear to be released into the culture medium of synthesising cells and is likely to be found bound to proteoglycans of the cell surface or extracellular matrix (Gotz et al., 1994). NT-6 is expressed in the developing and adult fish brain, as well as the adult gill, liver and eye. Weak expression is also found in the skin spleen, heart and skeletal muscle. Like NGF, NT-6 supports the survival of embryonic chick DRG and sympathetic neurons, although it is less potent than NGF (Gotz et al., 1994).

Neurotrophin-7 is the last member of the neurotrophin family to be identified (Lai et al., 1998; Nilsson et al., 1998). It possesses the 6 conserved cysteine residues as well as a 15aa acid insertion in the same location as that of NT-6. NT-7 is expressed at high levels in adult skin, heart and intestine and low levels in brain and testis (Lai et al., 1998). NT-7 promotes the survival and neurite outgrowth of chick DRG neurons (Lai et al., 1998).

1.4.1.5. The Trk family of receptors

Neurotrophins are capable of binding two types of receptor, the tyrosine kinase receptors of the Trk family and the unrelated p75 receptor. The Trk receptor was originally identified as a part of a proto-oncogene resulting from a DNA rearrangement fusing a truncated tropomyosin with a protein tyrosine kinase sequence (Martin-Zanca et al., 1986; Martin-Zanca et al., 1989). Now referred to as

trkA this gene encodes a 140kDa transmembrane glycoprotein containing a cytoplasmic tyrosine kinase domain capable of activating intracellular signalling pathways (Martin-Zanca et al., 1989). Two related genes have since been cloned and designated trkB and trkC, both encoding 145kDa proteins with 65-70% homology to the TrkA protein (Klein et al., 1989; Lamballe et al., 1991). The different trk receptors generally mediate the effects of different neurotrophins, NGF, BDNF and NT-3 preferentially binding and stimulating trkA, trkB and trkC respectively (Kaplan et al., 1991a; Kaplan et al., 1991b; Klein et al., 1991a; Klein et al., 1991b; Lamballe et al., 1991; Soppet et al., 1991; Squinto et al., 1991). Some cross-activation can occur with NT-3 also being able to bind trkA and trkB and NT-4/5 trkA and trkB (Berkemeier et al., 1991; Klein et al., 1991b; Klein et al., 1992; Soppet et al., 1991; Squinto et al., 1991), although higher levels of protein are usually required for these interactions. NT-7 has so far been reported to bind trkA with lower affinity, but the receptor for NT-6 has yet to be defined (Lai et al., 1998).

Trk receptors are activated through ligand-mediated dimerisation followed by autophosphorylation of the activation loop of the tyrosine kinase domain (Cunningham and Greene, 1998; Kaplan and Stephens, 1994). This autophosphorylation leads to increased activation of the catalytic portion of the receptors as well as revealing recruitment sites for protein substrates (Figure 1.1). A range of proteins, mediating signalling through a number of intracellular signalling pathways, are activated by Trk receptors, including Shc, PLC-γ1, SNT and SHP-1 (Belia et al., 1991; Borrello et al., 1994; Kaplan and Stephens, 1994; Obermeier et al., 1993; Ohmichi et al., 1991; Rabin et al., 1993; Vambutas et al., 1995; Dikic et al., 1995; Bibel and Barde, 2000). Shc activates PI3K and Ras/MEK/ERK, to promote neuronal differentiation and survival, while activation of PLC-γ1 activates PKC and increases intracellular free calcium. These increases in calcium levels particularly are associated with survival, injury and activity-dependent plasticity (Jiang and Guroff, 1997). SNT is implicated in cell cycle progression and SHP-1 is a protein phosphatase that regulates trophic factor receptor activation (Barbacid, 1994; Bartoe and Nathanson, 2000; Greene and Kaplan, 1995; Gunn-Moore and Tavare, 1998; Kaplan and Miller, 1997; Marsh et al., 2003).

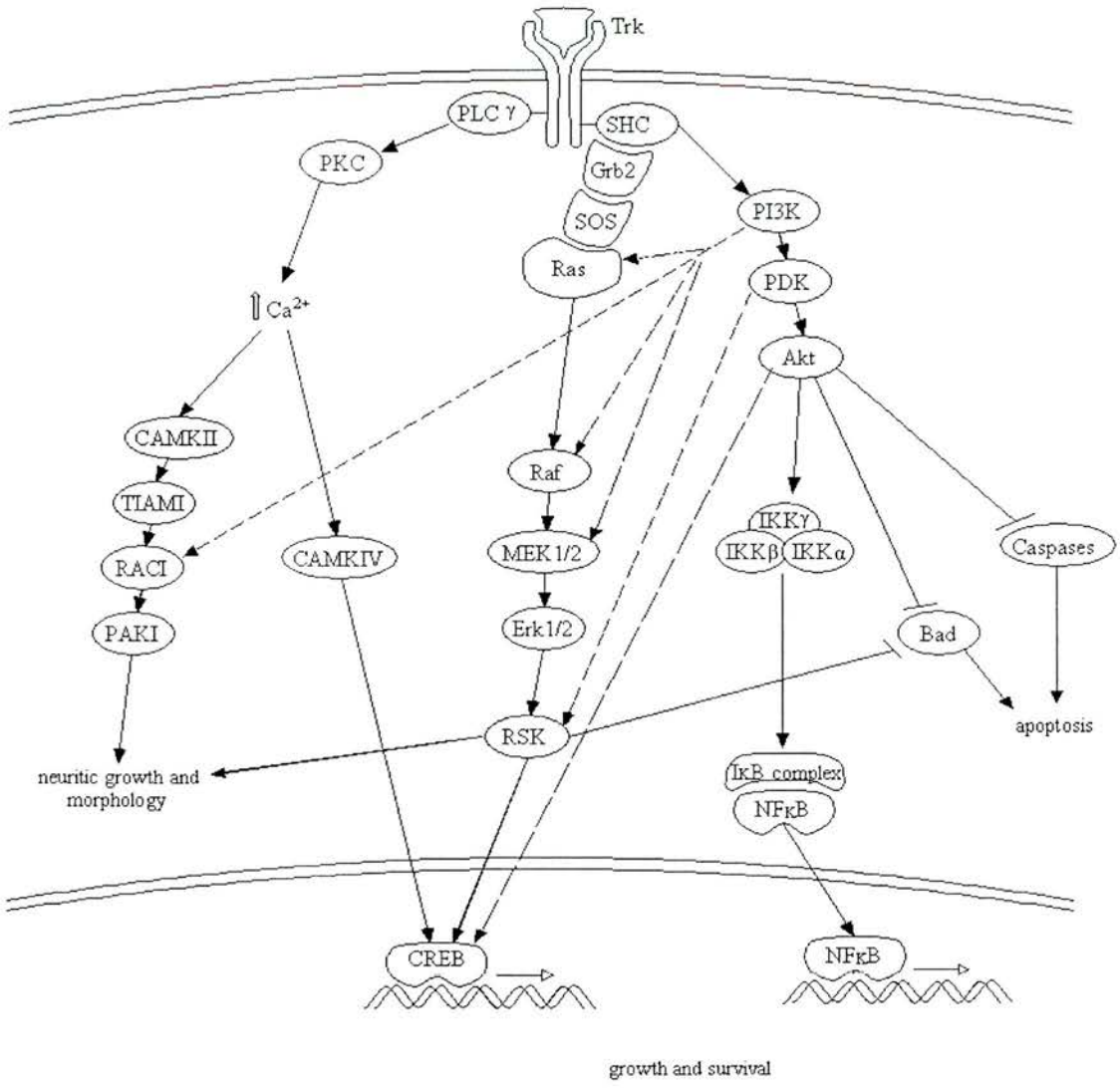

Figure 1.1 *Trk-activated signalling pathways*

This figure illustrates the principal pathways that are activated following neurotrophin-induced trk receptor dimerisation. This can result in the activation of the Ras/MEK/ERK, PI3 kinase and CAM kinase pathways. These pathways promote growth, survival and activity-dependant plasticity respectively, although, as can be see here, there is overlap between the pathways.

While cell bodies can be exposed to neurotrophins through autorcrine and paracrine mechanisms, as I described earlier a large part of neuronal exposure to neurotrophins is target derived. How then is a signal propgated from the distant nerve terminal to the cell body in order to effect changes in gene transcription? The current model describes retrograde transport of internalised, activated Trk receptors form the nerve terminal, through the axon, to the cell body by the transport of receptor containing vesicles. The Trk receptors carried by these vesicles are capable of activating signal transduction pathways along the entire length of the axon and in the cell body (Bhattacharyya *et al.*, 1997; Ehlers *et al.*, 1995; Kuruvilla *et al.*, 2004; Riccio *et al.* 1997). The signalling pathways activated by retrogradely transported Trk receptors differ in some respects to those activated at the site of neurotrophin exposure, for example retrograde signalling from NGF activated Erk5, rather than Erk1/2, on reaching the soma (Watson *et al.*, 2001).

Trk receptor expression is detected throughout the central and peripheral nervous systems. In the CNS, trkA expression is limited to cholinergic neurons of the basal forebrain and striatum (Holtzman et al., 1992; Merlio et al., 1992; Steininger et al., 1993; Vazquez and Ebendal, 1991), while the vast majority of central neurons express either trkB or trkC or both (Klein et al., 1989; Klein et al., 1990; Lamballe et al., 1994; Merlio et al., 1992; Tessarollo et al., 1993). In the periphery, trk receptors are detected on all neurons except parasympathetic ciliary neurons (Carroll et al., 1992; Klein et al., 1989; Lamballe et al., 1994; Martin-Zanca et al., 1990; Ninkina et al., 1996; Tessarollo et al., 1993) and in other non-neuronal neural crest-derived cell types (Schropel et al., 1995; Tessarollo et al., 1993). Outside the nervous system trkA expression also occurs in the immune system, trkB in lung muscle and ovary and trkC in facial structures as well as various tissues in the body cavity (Ehrhard et al., 1993; Lamballe et al., 1994; Tessarollo et al., 1993).

Mice carrying mutations in the trkA, B and C genes have been used to further elucidate the roles of neurotrophins during development. In general these mice have similar phenotypes to those of their dominant ligand null mutant (Crowley et al., 1994; Ernfors et al., 1994b; Ernfors et al., 1994a; Jones et al., 1994; Klein et al.,

1993; Klein et al., 1994; Smeyne et al., 1994). The principal difference is that mice lacking trkC have less severe defects that those of the NT-3 knockout mice, highlighting the more promiscuous binding of NT-3 to the Trk receptors and suggesting that its in vitro ability to signal through the other receptors is physiologically relevant. Mice lacking trkA display insensitivity to pain and have decreased neuronal populations in the trigeminal, dorsal root and sympathetic ganglia (Smeyne et al., 1994). TrkB-deficient mice lose a substantial proportion of trigeminal, nodose and DRG neurons (Klein et al., 1993), while trkC knockout mice have decreased numbers of myelinated axons emerging from the dorsal root and posterior columns of the spinal cord, as well as loss of a subpopulation of DRG neurons (Klein et al., 1994).

1.4.1.6. The p75 receptor

The p75 receptor is a second receptor for members of the neurotrophin family. Generally, it binds neurotrophins with a lower affinity that the Trk receptors, although high-affinity binding with NT-3 can also occur (Dechant et al., 1997). It has been identified in a range of species including human, rat and chicken (Chao et al., 1986; Large et al., 1989; Radeke et al., 1987). p75 is a 75kDa transmembrane glycoprotein containing a long extracellular domain with four cysteine repeats responsible for ligand binding (Welcher et al., 1991; Yan and Chao, 1991). These cysteine repeats are common to a range of cell surface receptors, such as Fas antigen and tumour necrosis factor (TNF) receptors I and II (Nagata and Golstien, 1995). Expression of p75 is detected in the developing and adult CNS (Ernfors et al., 1988; Yan and Johnson, Jr., 1989), as well as in developing sensory and sympathetic neurons, where it is upregulated once neurons have established target contact (Hallbook et al., 1990; Heuer et al., 1990; Wyatt et al., 1990).

The consequences of p75 activation vary considerably between cell types, and are affected by the presence or absence of trk receptors. p75 modifies the function of the high-affinity trk receptors (Barker and Shooter, 1994; Hempstead et al., 1991; Verdi et al., 1994) and can affect ligand discrimination by these receptors, particularly decreasing the ability of NT-3 to signal via TrkA (Benedetti et al., 1993; Bibel et al.,

1999; Brennan et al., 1999; Clary and Reichardt, 1994; Lee et al., 1994). p75 may also be able to activate its own intracellular signalling pathways, such as NFκB, to enhance neuronal survival in some sensory neurons (Hamanoue et al., 1999). Activation of NFκB by p75 is thought to be through interactions with the TRAF family of protein adaptors (Khursigara et al., 1999). This NFκB signalling appears to be activated by solely NGF, but not BDNF or NT-3, binding to p75 (Carter et al., 1996b; Hamanoue et al., 1999; Maggirwar et al., 1998). In contrast, p75 has also been found to promote apoptosis, both in the presence or absence of neurotrophins (Barrett and Bartlett, 1994; Frade and Barde, 1998; Rabizadeh et al., 1993). Evidence supporting the pro-apoptotic role of p75 can be found in BDNF knockout mice, where increased numbers of sympathetic neurons are found during development, despite BDNF not activating Trk receptors in these neurons (Bamji et al., 1998), and in mice overexpressing p75 where significant reductions in neuronal number in sympathetic, sensory and neocortical neurons are found, as well as increased cell death following axotomy (Majdan et al., 1997). Additionally, activation of p75 by NGF in TrkA-negative neurons promotes a cell death, which is abrogated by BDNF-activated TrkB signalling, suggests competitive actions of trk and p75 signalling pathways in cell survival (Davey and Davies, 1998).

Several adaptor proteins are recruited to the p75 receptor upon ligand binding and have been implicated in the pro-apoptotic effects of p75. Of these proteins NRIF (neurotrophin receptor interacting factor) appears particularly important in mediating cell death. Neuronal death in mice lacking the NRIF gene is decreased in a similar pattern to that of p75 and NGF knockout mice and may directly link p75 activation to transcription, as receptor activation leads to its nuclear translocation (Bibel and Barde, 2000; Casademunt et al., 1999). Other adaptor proteins that may be involved in p75-mediated apoptosis are NRAGE homolog (neurotrophin receptor interacting MAGE) and NADE (p75 NTR-associated cell death executor) (Mukai et al., 2000; Salehi et al., 2000) as well as p53 and the related protein p73 (Pozniak et al., 2000). Activation of p75 in the absence of Trk signalling may also act by increasing intracellular ceramide levels (Carter et al., 1996a; Casaccia-Bonnefil et al., 1996; Dobrowsky et al., 1994; Dobrowsky et al., 1995; Hamanoue et al., 1999). Ceramide

and JNK accumulation correlates with apoptotic signals in some cell types and inhibition of JNK activity can block apoptosis caused by activation of p75 (Brugg et al., 1996; Hartfield et al., 1998; Wiesner and Dawson, 1996; Yoon et al., 1998). p75 is also implicated in regulating neurite outgrowth through RhoA where p75 constitutively activated RhoA, with neurotrophin binding blocking this effect (Davies, 2000b; Yamashita et al., 1999; Yamashita and Tohyama, 2003). In contrast with the reported role of increased intracellular ceramide in mediating apoptosis, NGF-induced neurite outgrowth in hippocampal neurons has been shown to be through increased ceramide levels (Brann et al., 1999). Signalling of BDNF through p75 has also been reported to inhibit neurite outgrowth in postnatal sympathetic neurons, suggesting that there is much still to be learnt about neurotrophin signalling in neurite outgrowth.

1.4.2. Neuropoietic cytokines and their receptors

Within the nervous system, cytokines influence many processes including proliferation and survival, differentiation, myelination, plasticity and regeneration (English, 2003; Heller et al., 1996; Horton et al., 1996; Horton et al., 1998; Jonakait, 1997; Murphy et al., 1997; Profyris et al., 2004; Turnley and Bartlett, 2000). The neuropoietic cytokine family consists of nine proteins that are capable of signalling through the receptor protein, gp130. These are ciliary neurotrophic factor (CNTF), leukaemia inhibitory factor (LIF), oncostatin-M (OSM), cardiotrophin-1 (CT-1), interleukin-6 (IL-6) and interleukin-11 (IL-11), growth promoting activity (GPA), neuropoeitin (NP) and cardiotrophin-like cytokine (CLC, also known as novel neurotrophin-1/B-cell stimulating factor 3 or NNT-1/BSF-3). Sequence homology between these proteins is low, however all members carry a similar gene organisation and adopt similar tertiary structure, suggesting that they may have had a common ancestral gene (Bazan, 1991; Bruce et al., 1992).

1.4.2.1. Ciliary neurotrophic factor

CNTF is a 200-residue, highly acidic protein with a molecular mass of 20-24kDa (Masiakowski et al., 1991). It is the best characterised neuropoietic cytokine, and, unlike other family members such as LIF, is expressed exclusively in the nervous

system (Dobrea et al., 1992; Stockli et al., 1989; Stockli et al., 1991; Turnley and Bartlett, 2000). It was first identified as a protein capable of promoting the survival of parasympathetic neurons of the chicken ciliary ganglion in vitro (Adler et al., 1979) and has since been cloned in a number of mammalian systems including rabbit, rat, human and mouse (Kaupmann et al., 1991; Lam et al., 1991; Lin et al., 1989; Masiakowski et al., 1991; Stockli et al., 1989). In addition to ciliary neurons, a wide variety of peripheral and central neurons respond to CNTF *in vitro*, including neurons of the nodose, trigeminal, dorsal root and sympathetic ganglia, spinal motoneurons, hippocampal neurons and Purkinje cells (Arakawa et al., 1990; Barbin et al., 1984; Ernsberger et al., 1989; Horton et al., 1998; Ip et al., 1991; Larkfors et al., 1994). *In vivo*, CNTF is able to protect motoneurons and dopaminergic neurons, but not ciliary, sympathetic or sensory neurons, from naturally-occuring or axotomy-induced cell death (Curtis et al., 1993; Hagg and Varon, 1993; Oppenheim et al., 1991; Sendtner et al., 1990; Vejsada et al., 1995). Decreases in CNTF expression have also been associated with some familial amyotrophic lateral sclerosis and early onset of multiple sclerosis (Giess et al., 2002b; Giess et al., 2002a).

As well as its pro-survival effects, CNTF inhibits proliferation and induces cholinergic differentiation in developing sympathetic neurons and can influence neurotransmitter phenotype and receptor expression in a range of central and peripheral neurons (Burnham et al., 1994; Ernsberger et al., 1989; Louis et al., 1993a; Magal et al., 1991; Patterson and Nawa, 1993; Saadat et al., 1989; Wong et al., 1993; Zurn and Werren, 1994). CNTF also promotes neurite outgrowth in retinal ganglion cells and induces sprouting at the neuromuscular junction, but can inhibit dendritic outgrowth and cause dendritic retraction in sympathetic neurons where growth is promoted by BMP-7 (English, 2003; Goldberg et al., 2002; Guo et al., 1997; Guo et al., 1999; Gurney et al., 1992; Siegel et al., 2000; Tarabal et al., 1996). CNTF also acts on non-neuronal cells in the nervous system, promoting the differentiation of astrocyte progenitor cells and microglia *in vitro* (Bonni et al., 1997; Hughes et al., 1988; Rajan and McKay, 1998; Yoshida et al., 1993) as well as the maturation and survival of oligodendrocytes, depending on the culture conditions (Louis et al., 1993b; Mayer et al., 1994). Despite a number of roles for CNTF being

defined *in vitro*, its role *in vivo* is less well defined. The expression of CNTF mRNA is only detected postnatally (Ip et al., 1993; Sendtner et al., 1994; Stockli et al., 1991) and it appears only to be released from damaged cells, consequently CNTF null mutant display no overt phenotype at birth (Masu et al., 1993). It does, however, evidence a slow degeneration of a subset of adult motoneurons during the subsequent postnatal period, and in LIF/CNTF double knockout mice this degeneration is more severe than in either single knockout, suggesting redundancy in the system (Sendtner et al., 1996).

1.4.2.2. Leukaemia inhibitory factor

Unlike CNTF, LIF is widely expressed throughout the mammalian organism from the early stages of development, and has a large range of effects, particularly with respect to growth and differentiation (Turnley and Bartlett, 2000). At the earliest stages of development, LIF can suppress the differentiation of embryonic stem cells (Williams et al., 1988) and act as a survival factor for primordial germ cells (De Felici and Dolci, 1991; Matsui et al., 1992). LIF also plays many roles in haematopoiesis, inhibiting leukaemic cell production and stimulating the production of megakaryocytes and platelets (Metcalf et al., 1991; Metcalf et al., 1992). It also regulates bone resorption through osteoclasts (Martin et al., 1992).

In the peripheral nervous system, LIF can stimulate neural crest precursor cells to differentiate into sensory neurons and subsequently act as a survival factor for these neurons (Murphy et al., 1993; Murphy et al., 1994). In fact, a number of populations of embryonic and postnatal neurons survive when exposed to LIF *in vitro* including sensory neurons, parasympathetic ciliary neurons and neurons of the spiral ganglion (Adler et al., 1979; Horton et al., 1996; Horton et al., 1998; Marzella et al., 1997). LIF also regulates neurotransmitter phenotype and neuropeptide expression in motoneurons, neurons of the sympathetic ganglia and trigeminal sensory neurons (Fan and Katz, 1993; Michikawa et al., 1992; Nawa et al., 1991; Zurn and Werren, 1994). In the CNS, LIF can stimulate or inhibit differentiation of central neurons depending on the population and circumstances (Turnley and Bartlett, 2000). LIF promotes neurite outgrowth in spinal choliergic neurons and auditory neurons

(Gillespie et al., 2001; Richards et al., 1992), although it also inhibits dendritic growth of sympathetic neurons where neurite outgrowth is promoted by BMP-7 or NGF (Guo et al., 1997; Guo et al., 1999), and is associated with plasticity-related synaptic changes in primary sensory neurons and at the neuromuscular junction (English, 2003; Ramer et al., 1999; Thompson and Majithia, 1998). LIF is also important in promoting the generation, maturation and survival of oligodendrocytes and astrocytes *in vitro* (Chang et al., 2004; Kahn and De Vellis, 1994; Mayer et al., 1994; Nakagaito et al., 1995; Pitman et al., 2004; Richards et al., 1996; Yoshida et al., 1993). There are decreased numbers of GFAP-immunoreactive astrocytes in the hippocampus and dentate gyrus of LIF knockout mice (Koblar et al., 1998).

Like CNTF, LIF can maintain both motoneurons and peripheral sensory neurons neurons *in vivo* after nerve injury (Cheema et al., 1994b; Cheema et al., 1994a; Curtis et al., 1994). LIF also stimulates regeneration of olfactory neurons after injury and regenerative axonal growth in peripheral sensory neurons (Bauer et al., 2003; Cafferty et al., 2001; Moon et al., 2002).

1.4.2.3. Other neuropoietic cytokines

Oncostatin M (OsM) was originally described as inhibiting tumour cell proliferation (Zarling et al., 1986), however in the nervous system it supports the survival of peripheral sensory neurons *in vitro* (Horton et al., 1996) and promotes oligodendrocyte survival and differentiation (Vos et al., 1996). A subset of DRG neurons, those expressing both the vanilloid receptor-1 (VR-1) and P2X3, also express the OsM receptor, and these neurons are lost in $OsM^{-/-}$ mice (Morikawa et al., 2004; Tamura et al., 2003a). OsM is also implicated in regulating the expression of choline-acetyl transferase and vasoactive intestinal peptide expression in cultured sympathetic neurons (Rao et al., 1992). In the central nervous system the OsM receptor is expressed in the hypoglossal nucleus, as well as in olfactory bulb astrocytes (Tamura et al., 2003b).

Cardiotrophin-1 (CT-1) was characterised as a factor that mediates cardiac hypertrophy (Pennica et al., 1995a). Within the nervous system, it is a survival factor

for cultured sensory and parasympathetic neurons, hindbrain dopaminergic neurons and motoneurons (Arce et al., 1998; Horton et al., 1998; Pennica et al., 1995b; Pennica et al., 1996a).

IL-6 promotes the survival of cultured embryonic and postnatal midbrain catecholaminergic neurons and basal forebrain cholinergic neurons, as well as stimulating astrocyte proliferation (Fattori et al., 1995; Hama et al., 1989; Kushima et al., 1992; Kushima and Hatanaka, 1992a; Kushima and Hatanaka, 1992b). Additionally, IL-6 is a survival factor for peripheral sensory neurons in culture (Horton et al., 1996).

Cardiotrophin-like cytokine (CLC) was identified in 1999 as a novel ligand for the CNTFRα/LIFRβ/gp130 receptor complex (Senaldi et al., 1999; Shi et al., 1999). CLC is expressed mainly in secondary lymphoid organs, but has also been detected in a number of other systems, including kidney, heart, lung and pancreas (Senaldi et al., 1999; Shi et al., 1999). It appears to be released from cells only in a complex with a protein called cytokine-like factor 1 (CLF-1), a soluble protein that has a high level of homology with gp130 (Elson et al., 1998; Elson et al., 2000), or with a soluble form of the CNTFRα (Plun-Favreau et al., 2001). In the nervous system CLC supports the survival of embryonic motor and sympathetic neurons (Elson et al., 2000; Plun-Favreau et al., 2001; Senaldi et al., 1999) and can induce astrocyte differentiation (Uemura et al., 2002).

Neuropoietin is a recently identified cytokine that acts through the CNTFRα/LIFRβ/gp130 complex to activate STAT3 signalling (Derouet et al., 2004). Neuropoietin is highly expressed during embryonic development, a stage at which the alternate CNTFRα ligands CNTF and CLC are not present. It is expressed in embryonic neuroepithelial cells, the retina, and skeletal muscle and *in vitro* promotes the survival of embryonic motoneurons, as well as promoting the proliferation of neural precursors in association with epidermal growth factor (EGF) and fibroblast growth factor (FGF) 2.

1.4.2.4. The neuropoietic cytokine receptor complex.

Neuropoietic cytokines signal through a multicomponent receptor complex. This consists of a common subunit, a glycoprotein called gp130, which interacts with different adaptor proteins depending on the ligand involved (Heinrich et al., 2003; Hibi et al., 1990; Sato and Miyajima, 1994). LIF binds directly to a receptor subunit called LIF receptor β (LIFRβ), which induces dimerisation with the gp130 subunit, while CNTF, neuropoietin and CLC require an additional protein, CNTFRα, to induce the formation of a complex with first gp130 then LIFRβ (Davis et al., 1991; Davis et al., 1993; Gearing et al., 1991; Gearing et al., 1992; Ip et al., 1993; Stahl and Yancopoulos, 1994) (Elson et al., 2000; Plun-Favreau et al., 2001). CT-1 also appears to require an adaptor protein to interact with gp130 and LIFRα, but this protein is yet to be identified (Pennica et al., 1995a; Pennica et al., 1996b; Robledo et al., 1997). IL-6 and IL-11 have their own supplementary subunits, IL6Rα and IL11Rα (Barton et al., 2000; Murakami et al., 1993), and ligand binding induces gp130 homodimerisation as part of a hexameric complex while OSM induces the heterodimerisation of gp130 and the OSM receptor subunit (OSMR) (Heinrich et al., 2003; Ichihara et al., 1997; Lindberg et al., 1998).

Neither gp130 nor LIFRα themselves possess intrinsic enzymatic activity, thus ligand binding induces a conformational change in the receptor complex that results in the phosphorylation and activation of receptor tyrosine kinases that are associated with LIFRβ and gp130 even in the inactive state (Stahl and Yancopoulos, 1994). The receptor tyrosine kinases typically associated with the gp130 receptor complexes are of the janus kinase, or JAK, family, particularly JAK1 (Vlotides et al., 2004). Activated JAKs then phosphorylate several tyrosine residues in the cytoplasmic tails of gp130 and LIFRβ, revealing docking sites for proteins containing SH2-domains including SHP-2, Shc and Grb, which link cytokine signalling to the MAPK pathway, PLCγ, PI3K, and Raf-1 (Boulton et al., 1994; Chen et al., 1999; Giordano et al., 1997; Lelievre et al., 2001; Oh et al., 1998; Stahl et al., 1994; Stahl et al., 1995; Stahl and Yancopoulos, 1994). The activation of PI3K and Akt by cytokines has been implicated in pro-survival responses to cytokines in both central (motor) and peripheral (nodose) neurons (Alonzi et al., 2001; Nishimune et al., 2000). Ligand

binding also results in the activation of the STAT family of SH2-bearing proteins, particularly STAT-3, which appears to be the principal pathway for cytokine signalling (Segal and Greenberg, 1996; Stahl and Yancopoulos, 1994; Turnley and Bartlett, 2000; Zhong et al., 1994). Activated STAT proteins dimerise and translocate to the nucleus where they activate the transcription of a number of genes (Chen et al., 1999; Heinrich et al., 2003; Hirano et al., 2000). These genes include a number of anti-apoptotic proteins such as Bcl-2 and Bcl-xL (Catlett-Falcone et al., 1999; Chen et al., 1999; de Koning et al., 2000; Fukada et al., 1996; Karni et al., 1999) as well as the neurotrophic factor Reg-2 (Nishimune et al., 2000).

As each receptor subunit is used in receptor complexes formed by a number of ligands, the phenotypes of mice lacking each receptor subunit are more severe than those of mice with null mutations for any of the ligand genes. CNTFRα knockout mice display the mildest phenotype, surviving to birth, however these mice die shortly after birth, failing to initiate the feeding process, and showing a dramatic loss of motoneurons (DeChiara et al., 1995). This phenotype is more severe than that of the CNTF ligand knockout mice and, as the CNTFRα is expressed strongly long before expression of CNTF is detected (Ip et al., 1993; Sendtner et al., 1994), this led to the proposal that another ligand "CNTF2" must signal through CNTFRα. Recent candidates for "CNTF2" have recently been found to be neuropoeitin and CLC (Derouet et al., 2004; Elson et al., 2000; Plun-Favreau et al., 2001; Senaldi et al., 1999; Shi et al., 1999). LIFRβ knockout mice also die within hours of birth, with significant reductions in the number of facial, trigeminal hypoglossal and spinal motoneurons and a loss of astrocytes in the brainstem and spinal cord (Li et al., 1995; Ware et al., 1995). In $gp130^{-/-}$ knockout mice there is loss of DRG neurons and specific populations of motoneurons, that occurs between E14.5 and E18.5, suggesting that cytokines are principally involved in regulating neuronal survival during the period of naturally occurring cell death, rather than in the early generation of neurons. $Gp130^{-/-}$ mice also show a significant loss of astrocytes (Nakashima et al., 1999).

1.4.3. The reg family of proteins

The Reg family of proteins is a large family of secretory proteins, so named because the first Reg gene was identified as a gene upregulated in regenerating pancreatic β-cells (Terazono et al., 1988). Since then five further Reg family members have been identified in mice, separated into 3 different groups based on their structural homology (Abe et al., 2000; Narushima et al., 1997; Unno et al., 1993). Over the same period of time several Reg-family proteins have also been isolated from other mammalian cells types. Some of these proteins are homologues of the murine reg genes and fit into the three murine groups, while the identification of an additional

Table 1: The Reg family of proteins

	MOUSE	*RAT*	*HUMAN*	*OTHER NAMES*	*REFERENCES*
Group I	Reg-I	Reg-I	Reg-Iα Reg-Iβ	PSP/PTP/ lithostathine Reg-H	(Moriizumi et al., 1994; Okamoto, 1999; Unno et al., 1993)
Group II	Reg-II				(Unno et al., 1993)
Group III	Reg-IIIβ/ Reg-2 Reg-IIIα RegIIIγ RegIIIδ	PAP-I PAP-II PAP-III	PAP Reg-III	HIP/Peptide 23 INGAP	(Abe et al., 2000; Baeuerle and Baltimore, 1988; Frigerio et al., 1993; Iovanna et al., 1991; Katsumata et al., 1995; Lasserre et al., 1992; Narushima et al., 1997; Nata et al., 2004; Orelle et al., 1992; Rafaeloff et al., 1997; Suzuki et al., 1994; Taylor-Fishwick et al., 2003)
Group IV			Reg-IV		Hartupee et al., 2001

human protein has led to a fourth group being established (Hartupee et al., 2001). The converging identifications of different Reg-family proteins in different species have led to a complicated nomenclature (see Table 1). For the purposes of this thesis the murine nomenclature will generally be used, with regIIIβ/Reg-2 being referred to as Reg-2 throughout.

Each reg family gene covers approximately 3kbp, arranged into 6 exons and 5 introns with identical intron/exon boundaries, an organisation which is conserved across different species (Abe et al., 2000; Hartupee et al., 2001; Narushima et al., 1997; Unno et al., 1993). With the exception of the Reg-IV in humans, these genes are clustered in the same chromosome loci, 2p12 in humans or 6C in mice, and are consequently considered to have arisen through gene duplication (Okamoto, 1999). Reg-family proteins are expressed in varying ranges of tissues and play multiple roles, functioning particularly as anti-apoptotic, growth factors or mitogens for pancreatic β cells, neural cells or gastrointestinal epithelial cells (Zhang et al., 2003).

Group III is the only group whose members have been found to be active in the nervous system (Livesey et al., 1997; Nishimune et al., 2000; Schweizer et al., 2002; Tam et al., 2002). Of the four murine group III genes, both PAP/Reg-IIIβ/Reg-2 and INGAP/Reg-IIIδ are known to function in the nervous system. Livesey et al (1997) first reported a role for Reg-2 in the nervous system when they detected its expression in developing or regenerating motor neurons or sensory neurons of the DRG. They found Reg-2 to be a potent Schwann cell mitogen, and that blocking Reg-2 signalling significantly delayed axonal regeneration, which is itself dependent on Schwann cell proliferation. Reg-2 expression during development is dependent on contact with peripheral target tissues, with the IL-6 type cytokines probably mediating this effect, as Reg-2 expression is abolished in mice lacking the LIF receptor gene. The expression of Reg-2 in regenerating DRG neurons at different time points varies depending on the subtype of neurons, with small-diameter nociceptive neurons expressing Reg-2 24h after transection, while after 5 days Reg-2 expression is predominantly in large diameter non-nociceptive neurons (Averill et al., 2002). The induction of Reg-2 expression in axotomised motor neurons is dependent on functional STAT-3 signalling (Schweizer et al., 2002). STAT-3 is a

major downstream mediator of IL-6-type cytokine signalling therefore this suggests another link between cytokines and Reg-2 expression in neurons. In developing motor neurons *in vitro*, Reg-2 is an indispensable intermediate for CNTF-induced neuronal survival. Reg-2 promotes the survival of these neurons via a PI3K- and NFκB-dependent pathway (Nishimune et al., 2000). In motoneurons *in vivo*, Reg-2 expression occurs after innervation of the target muscle.

Group III reg proteins have also been implicated in neurite outgrowth in the peripheral nervous system. In adult DRG explants, a model of nerve axotomy, the 15 amino acid active sequence of INGAP/Reg-IIIδ enhances neurite outgrowth in a dose-dependent manner (Tam et al., 2002). This effect was, however, blocked by a preincubation of the explant with anti-NGF antibodies, a treatment which itself does not independently alter neurite outgrowth, suggesting that INGAP peptide may stimulate NGF production within the ganglion. INGAP peptide stimulated $[H]^3$ thymidine incorporation into these explants, suggesting that it was acting on a population of proliferating non-neuronal cells in the ganglia.

In contrast to the growth-promoting role described above, Reg-2 has been implicated in the aetiology of Alzheimer's Disease (AD). Reg proteins are highly susceptible to proteolysis, producing a C-terminal polypeptide that forms insoluble fibrils at physiological pH (Cerini et al., 1999) reminiscent of Aβ and Tau in senile plaques and neurofibrillary tangles in AD. Reg-I and Reg-2 are found in such protein deposits in AD (Duplan et al., 2001). The levels of both proteins are also elevated in the cerebrospinal fluid in AD patients. Interestingly, cytokines such as TNF-α and IL-6, which are capable of regulating Reg-family protein expression, have also been implicated in AD (Fillit et al., 1991; Ringheim et al., 1998; Singh and Guthikonda, 1997) with this synthesis of TNF-α by microglia being stimulated by Aβ peptides. Duplan et al. (2001) have speculated that the production of Reg proteins and their subsequent precipitation contribute to a cycle that worsens precipitate formation in AD plaques and tangles.

Reg family gene expression is constitutive in some tissues, such as the pancreas and the developing pituitary gland, through the presence of DNA binding motifs for

tissue-specific transcriptional regulators such as Pan-1 and Pit-1 in their promoter sequences (Abe et al., 2000; Narushima et al., 1997; Vierra and Nelson, 1995). Reg family genes are also induced in response to cytokine exposure, with the promoter region of each gene containing a variety of IL-6 response elements (IRE) (Abe et al., 2000; Dusetti et al., 1995; Narushima et al., 1997; Okamoto, 1999). Expression of Reg-I in pancreatic islet β cells can be induced by IL-6, growth-hormone releasing hormone, TNF-α and interferon-γ (Chakraborty et al., 1995; Dusetti et al., 1996; Okamoto, 1999). Reg-2 mediates effects of TNF-α in pancreatic acinar cells, and its expression is induced by IL-6 in combination with dexamethasone, TNF-α or IFN-γ in these cells (Dusetti et al., 1995). Reg-2 has also been reported to be induced by growth-hormone releasing hormone in the small intestine and pituitary and oestradiol in the uterus (Chakraborty et al., 1995; Katsumata et al., 1995).

Reg-I is the only member of the reg family of proteins for which a receptor has been described. Isolated by probing a rat islet cDNA expression library with $[I]^{125}$-labelled Reg I, the 919 amino acid sequence predicts a type II transmembrane protein with a long extracellular domain (868 a.a.) and a short intracellular N-terminal (28 a.a.) (Kobayashi et al., 2000). The amino acid sequence has 97% homology to the human Ext-like gene 3 (Extl3) sequence, suggesting that Extl3 is the rat homologue of this protein. Expression of this protein in COS-7 cells confers Reg-1 binding and overexpression of this putative receptor in pancreatic islet cells increased proliferation in response to Reg-I.

1.5. Intracellular Signalling

Intracellular signalling is a diverse and rapidly expanding field of study. One simple way of classifying signalling cascades types is into those that use non-protein intermediates or second messengers (such as calcium ions, cyclic nucleotides or phosphoinositides) to exert their physiological actions and those that do not, such as G-protein based systems or those based on using phosphorylation of specific residues in subsequent proteins to modify to their functions. These are not, however, discrete systems since signalling pathways using second messengers will usually use G-

proteins and protein kinases in their signalling cascades, and there is extensive potential for crosstalk between different signalling cascades.

Individual signalling cascades are usually identified by significant proteins that occur at some point in the cascade. These names can vary from second-messenger activated proteins, to a particular transcription factor. The signalling pathway that is the major focus of investigation in this thesis is that centred on the NFκB family of transcription factors. Several other pathways are also considered, as they are activated by the growth factors used, or commonly associated with signalling for either neuronal survival or outgrowth. These pathways are the JAK/STAT signalling pathway, commonly implicated in mediating the intracellular effects of cytokines, and the PI3 kinase/Akt and MAP kinase pathways, common pathways mediating neurotrophin signalling. In this section of the introduction I will describe the structure of the NFκB signalling pathway. I will then discuss the roles of these signalling pathways neuronal survival and outgrowth.

1.5.1. The nuclear factor κB family of transcription factors

Nuclear factor κB (NFκB) was first described almost 20 years ago by (Sen and Baltimore, 1986) as a protein that binds an immunoglobulin κ enhancer sequence. Since then NFκB family proteins have been found to be important in signal transduction in a wide variety of situations, particularly in the immune response and other times of cell stress, often by helping support cell survival (Li and Stark, 2002).

NFκB family proteins are highly conserved, from Drosophila to humans, with each member containing a characteristic Rel homology (RH) domain. Structurally, NFκB family proteins are divided into two groups, the first group containing the mammalian proteins, p65 (Rel A), Rel (c-Rel) and Rel B as well as the drosophila proteins Dorsal and Dif, while the second group contains p100, p105 and Relish (Siebenlist et al., 1994). All these proteins contain the RH domain, with additional regions varying between the two groups and determining their functions. Proteins from the group containing p65 possess c-terminal transcriptional activation domains. Members of the second group possess 5-7 ankyrin repeats in long c-terminal domains that act to inhibit nuclear translocation. Before signalling can occur p100 and p105

undergo partial degradation, p100 to a protein called p52 and p105 to p50, releasing proteins that are able to participate in the activation of transcription.

Transcriptionally active NFκB family proteins form homo- or hetero-dimers, the most common of which is the p65-p50 dimer, often referred to simply as NFκB, its original name (Li and Stark, 2002). In the inactive state, NFκB dimers are bound by a protein complex called IκB or consist of a dimer containing the p52/p50 precursor proteins p100 or p105 (Baeuerle and Baltimore, 1988). The IκB complex is formed from combinations of 5 subunits, IκBα, IκBβ, IκBγ, IκBε and Bcl-3, with IκBα and IκBβ being the principal proteins (Ghosh et al., 1998; Ghosh and Karin, 2002; Silverman and Maniatis, 2001). IκB proteins are characterised by the presence of C-terminal ankyrin repeats that mediate their interaction with NFκB. IκBα also contains a nuclear export sequence (NES) and is involved in removing NFκB from the nucleus (Tam et al., 2000). The IκB complex conceals several important regions of the NFκB subunits, including the nuclear localisation sequence of p65, as well as regions important for DNA binding and phosphorylation sites necessary for maximal activation of gene transcription (Hayden and Ghosh, 2004). For a long time it was thought that the IκB complex limited NFκB to the cytosol, however it has recently been discovered that while NFκB is held inactive by the IκB complex, the p50 nuclear localisation signal is still exposed and IκB/NFκB complexes are constantly being transported into the nucleus. The nuclear export sequence of IκBα, however, causes the removal of inactive dimers, resulting in a situation where inactive NFκB/IκB complexes are constantly being shuttled into and out of the nucleus, but where the balance of this shuttling leaves the majority of NFκB dimers in the cytoplasm at any one time (Huang et al., 2000b; Huxford et al., 1998; Johnson et al., 1999; Malek et al., 2001; Malek et al., 2003). In order for the NFκB become transcriptionally active the principal event that must occur is for the NFκB dimer to be released from the IκB complex. This removes the contribution of the NES of IκBα to the equilibrium, leading to an accumulation of NFκB dimers in the nucleus, and exposes the p65 nuclear localisation signal, DNA binding and phosphorylation sites (Huang et al., 2000b; Huxford et al., 1998; Johnson et al., 1999; Malek et al., 2001; Malek et al., 2003).

There are several ways in which dissociation of NFκB from the IκB complex can be initiated (figure 1.2). The principal method is serine phosphorylation of an IκB protein, on serines 32 and 36 for IκBα or Ser 19 and Ser 26 in IκBβ. This allows the IκB protein to be recognised by the βTrCP subunit of the SCF ubiquitin ligase complex, targeting it for proteosomal degradation, and is the classical pathway of NFκB activation (Karin and Ben Neriah, 2000). More recently, additional events leading to the release of active NFκB dimers have been described. Phosphorylation of IκB at tyrosine residues has been described as a mechanism initiating the release of NFκB dimers from the IκB complex. This mechanism appears to be used particularly in response to oxidative stress (Fan et al., 2003; Mahabeleshwar and Kundu, 2003; Natarajan et al., 2002; Takada et al., 2003). Phosphorylation of this residue leads of dissociation of NFκB from the IκB complex, but appears to be independent of IκB degradation (Canty et al., 1999; Natarajan et al., 2002; Takada et al., 2003). This mechanism has been found to be activated in response to NGF exposure in neuronal cell lines, as well as primary hippocampal neurons (Bui et al., 2001). A third pathway leading to the release of functioning NFκB dimers occurs when the RelB protein is associated with the inactive p100. Phosphorylation of the p100 protein on at least 5 serine residues (serines 99, 108, 115, 123, and 872) targets it for partial degradation releasing a p52/RelB dimer (Dejardin et al., 2002; Xiao et al., 2001; Xiao et al., 2004).

A number of different kinases are capable of phosphorylating IκB proteins, thus releasing NFκB for signal transduction. The protein kinases that perform this service as part of the classical activation cascade are those of the IκB kinase (IKK) complex. There are three members of this class of protein, IKKα, IKKβ and IKKγ (also called NEMO; NFκB Essential Modifier). IKKα and IKKβ both possess catalytic ability while the third member, IKKγ, which is catalytically inactive, regulates the formation of the IKK complex and mediates many of the upstream signals that result in IKK activation (May et al., 2000; May et al., 2002; Rudolph et al., 2000; Yamaoka et al., 1998). The IKK complex is formed from either IKKα or IKKβ homodimers, or IKKα/β heterodimers, regulated by IKKγ. The IKKα/β heterodimer has the highest catalytic activity *in vitro* (Huynh et al., 2000), however the two subunits do not have

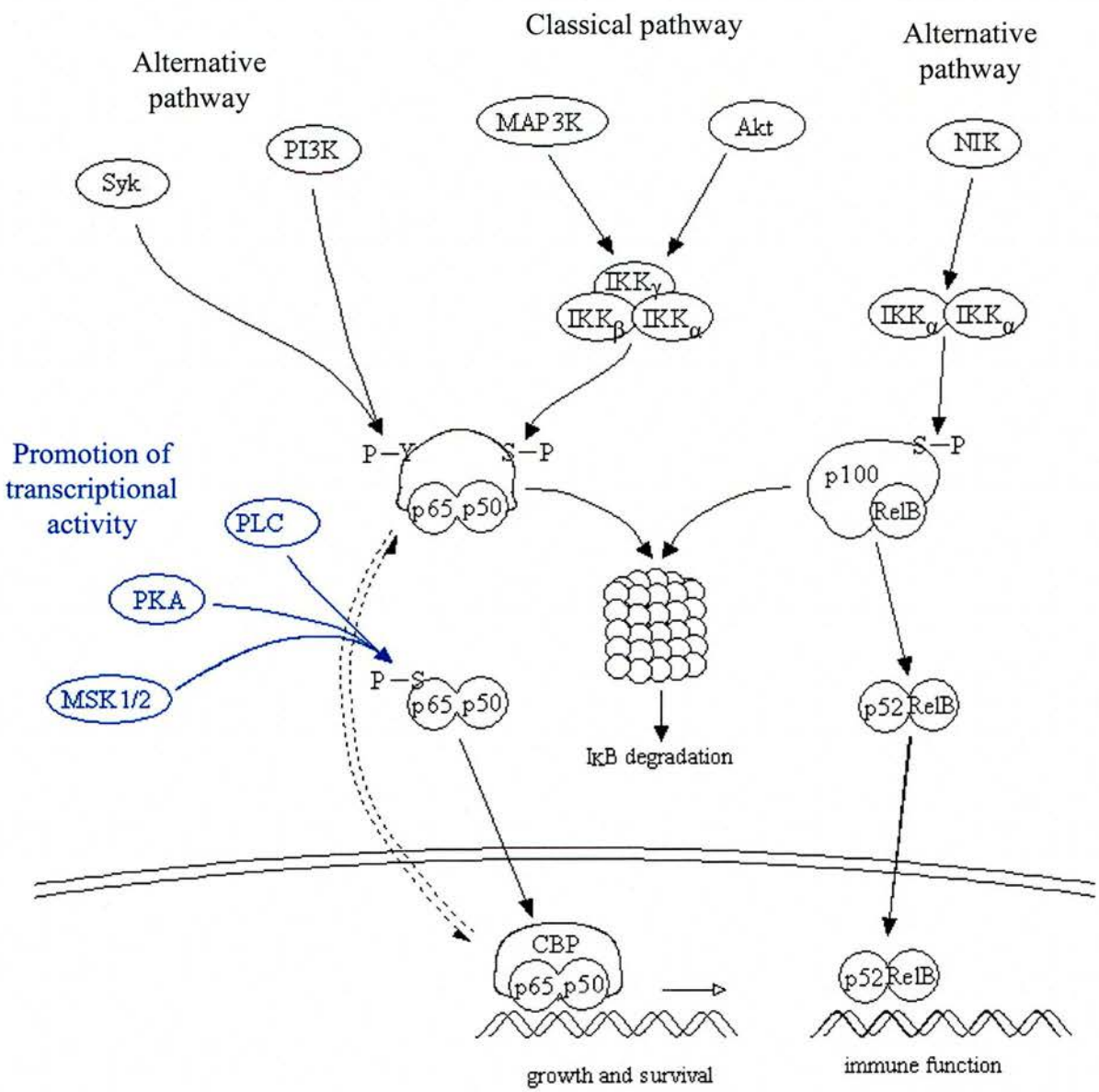

Figure 1.2 *Intracellular pathways leading to NFκB activation.*

This figure illustrates the principal pathways leading to NFκB activation. Phosphorylation activates the IKK complex, leading to IκB phosphorylation on serine residues and its subsequent degradation, releasing p50-p65 dimers for nuclear translocation and transcriptional activation. Tyrosine phosphorylation of IκB does not result in its degradation, but nevertheless releases p50-p65 dimers and is one alternative pathway. A second alternative pathway invloves activation of IKKα, leading to the phosphorylation and partial degradation of p100 and releasing p52-RelB dimers. Serine phosphorylayion of p50-p65 dimers can also increase its transcriptional activity and its ability to associate with its coactivator CBP.

identical functions. IKKβ is the protein kinase that necessary for serine phosphorylation of IκBα and IκBβ, leading to activation of NFκB through the classical pathway and cannot be replaced by IKKα (Hayden and Ghosh, 2004). Whether IKKα is involved in the classical pathway is still unknown, although recent evidence suggests that IKKα, but not IKKβ or IKKγ, has a nuclear role, phosphorylating histone H3, a process that is important for NFκB-dependent gene transcription (Anest et al., 2003; Birbach et al., 2002; Yamamoto et al., 2003). IKKα is also the kinase involved in the non-canonical pathway, where phosphorylation of p100 leads to its partial degradation, leaving RelB-p52 dimers (Solan et al., 2002; Xiao et al., 2001; Xiao et al., 2004; Yilmaz et al., 2003). Additionally, IKKα appears to have some NFκB independent actions, particularly involved in the formation of the epidermis (Cao et al., 2001; Hu et al., 1999; Hu et al., 2001; Li et al., 1999a; Takeda et al., 1999). The differences in the roles of the IKKα and β proteins are reflected in mice lacking these genes. The phenotype of the $IKK\beta^{-/-}$ mice resembles that of $p65^{-/-}$ and both mutations are lethal by E15 (Beg et al., 1995; Li et al., 1999b; Li et al., 1999c; Tanaka et al., 1999). In contrast, $IKK\alpha^{-/-}$ embryos survive until birth, but have significant epidermal and skeletal defects that result in death soon after birth, and which are not dependent on NFκB activation (Cao et al., 2001; Hu et al., 1999; Hu et al., 2001; Li et al., 1999a; Sil et al., 2004; Takeda et al., 1999). Like IKKβ and p65-deficient mice, $IKK\gamma^{-/-}$ mice die from liver apoptosis around E13 (Rudolph et al., 2000).

Several models have been proposed for activation of the IKK complex. In one case IKK activity occurs through serine phosphorylation by MAP kinase kinase kinases (MAP3K), while in another the IKK complex is recruited to cell surface receptors, where autophosphorylation occurs. Several MAP3Ks have been found to associate with the IKK complex and *in vitro* can activate the IKK complex. These include NFκB-inducing kinase (NIK), mitogen-activated protein/Erk kinase kinase (MEKK) 1, MEKK 3, TGFβ activating kinase 1 (TAK1) and NFκB activating kinase (NAK) (Lee et al., 1998; Ninomiya-Tsuji et al., 1999; Tojima et al., 2000; Woronicz et al., 1997; Yang et al., 2001). The physiological significance of several of these interactions, particularly those of NIK and MEKK1 in the classical activation of

NFκB have proved hard to substantiate, particularly using gene knockouts. This effect could be a consequence of redundancy among MAP3 kinases (Yamamoto and Gaynor, 2004; Yin et al., 2001; Yujiri et al., 2000) or alternatively because some of these proteins are being found alternative roles as adaptor proteins (Hayden and Ghosh, 2004). One MAP3K, NIK, does play a role in activating NFκB dimers, through the non-canonical NFκB pathway, where it activates IKKα, leading to partial degradation of p100 and release of RelB-p52 dimers (Claudio et al., 2002; Coope et al., 2002; Dejardin et al., 2002; Lin et al., 1998b; Ling et al., 1998; Muller and Siebenlist, 2003; Regnier et al., 1997; Senftleben et al., 2001; Uhlik et al., 1998; Xiao et al., 2001; Yilmaz et al., 2003). NIK activation in the non-canonical pathway is caused by factors such as B cell activating factor and CD40 ligand (Claudio et al., 2002; Coope et al., 2002) Polyubiquitination of IKKγ through a TRAF6/TAK interaction also seems to positively regulate NFκB signalling (Deng et al., 2000; Tang et al., 2003; Wang et al., 2001). Less is known about the induction of tyrosine phosphorylation of IκB proteins than about serine phosphorylation. It has been suggested that PI3K may be involved in neurotrophin-activated tyrosine phosphorylation, and the Syk tyrosine kinase in hydrogen peroxide-induced NFκB activation (Beraud et al., 1999; Bui et al., 2001; Takada et al., 2003).

As well as release from the IκB complex, several modifications to the NFκB proteins regulate their transcriptional activity. Such modifications alter the ability of NFκB to associate with other elements, including DNA and co-activator proteins. NFκB subunits p65 and c-Rel can be phosphorylated on serine 276 by PKA, which enhances DNA binding and exposes an site for interaction with CBP/p300, a transcriptional co-activator (Naumann and Scheidereit, 1994; Neumann et al., 1995; Zhong et al., 1997; Zhong et al., 1998; Zhong et al., 2002). Other protein kinases also perform this function, including MSK1 and 2 (Vermeulen et al., 2003). Serine phosphorylation on other residues also affects transcriptional activation. Both IKKα and IKKβ have been shown to phosphorylate p65 at ser 536 (O'Mahony et al., 2004; Sizemore et al., 2002; Yang et al., 2003) while PKCζ phosphorylates p65 at Ser 311 (Anrather et al., 1999; Leitges et al., 2001). GSK3 and TBK1 are also implicated in

NFκB signalling, although whether they directly modify NFκB proteins is as yet unknown (Bonnard et al., 2000; Hoeflich et al., 2000; Schwabe and Brenner, 2002).

The different NFκB proteins are not interchangeable. The release of different active dimers has varying biological effects, with particular dimers being implicated in the signalling of specific factors in different cell types. The most basic indication of the roles of each of the NFκB proteins can be gained from transgenic mice. Mutants lacking the p65 gene reveal the essential role of NFκB in preventing apoptosis during development, as $p65^{-/-}$ embryos dies from liver degeneration at E15-16 (Beg et al., 1995). Rescue of this defect by crossing with the mice deficient in TNFα then reveals increased susceptibility to infection and apoptosis in some neuronal populations (Alcamo et al., 2001; Middleton et al., 2000). Mice lacking the p50 gene show a very different phenotype. While there are no major developmental defects, there are major defects in immune system function (Sha et al., 1995). Defects in the immune system also occur in p52, c-Rel and RelB knockout mice (Caamano et al., 1998; Franzoso et al., 1998; Paxian et al., 2002). The differences in the roles of NFκB dimers are partly related to differences in the ability of different dimers to bind to each other and the IκB proteins. IκBα associates mainly with p65/p50, IκBε with p65 homodimers and c-Rel/p65 dimers, and Bcl-3 with p50 or p52 homodimers, while RelB can interact only with p100 (Bours et al., 1993; Dobrzanski et al., 1994; Fujita et al., 1993; Hatada et al., 1992; Hayden and Ghosh, 2004; Li and Nabel, 1997; Ryseck et al., 1992; Simeonidis et al., 1997; Solan et al., 2002; Whiteside et al., 1997). Not all possible combinations of NFκB hetero- and homo- dimers occur *in vivo*. RelB, for example, only interacts with p50 and p52, while homodimers of p50 and p52 bind to NFκB sequences but, having no inherent transcriptional domain, usually have an inhibitory effect on gene transcription (Ishikawa et al., 1998; Li and Verma, 2002; Zhong et al., 2002). Different combinations of NFκB subunits activate transcription of different sets of genes depending on the dimer, with some genes being present in the activation sets of more than one dimer (Dejardin et al., 2002; Saccani et al., 2003). This specificity is partly embedded in the different κB binding sequences, as different sequences bind NFκB dimers with varying efficiencies. Binding efficiency,

however, does not necessarily correlate with transcriptional activity and is also modified by other elements in the promoter sequences (Saccani et al., 2003).

Genes transcribed as a consequence of NFκB activation include cytokines, such as TNFα, IL-6 and GM-CSF, chemokines, adhesion molecules, such as ICAM-1 and inducible enzymes, such as iNOS and COX-2 (Yamamoto and Gaynor, 2004). NFκB also regulates the expression of receptors involved in immune recognition, proliferation and cell survival. Proteins involved in regulating cell survival include cellular inhibitors of apoptosis (c-IAP 1 and 2), TNF-receptor associated factors (TRAF 1 and 2) and Bcl-2 family genes (Karin and Lin, 2002; Yamamoto and Gaynor, 2004).

1.5.2. Intracellular signalling and the regulation of neuronal survival in the developing nervous system

The survival of any cell depends on a "decision" at the intracellular level as to whether the intrinsic apoptotic program will be executed. Thus, proteins that promote cell survival activate intracellular signalling pathways that alter the balance of the pro- and anti-apoptotic cellular machinery to support survival. A number of intracellular signalling pathways have been implicated in this effect in a variety of systems, the principal of which is the PI3K/Akt pathway. PI3K and Akt (also called protein kinase B) are two proteins that together form part of a major pathway that regulates neuronal survival using both transcription-dependent and independent mechanisms (Brunet et al., 2001; Datta et al., 1999). Neurotrophic factors, such as neurotrophins and cytokines, on activating their receptors, recruit PI3K to the plasma membrane, where it catalyses the production of phosphoinositide phosphates PIP_2 and PIP_3 (Alonzi et al., 2001; Crowder and Freeman, 1998; Hetman et al., 1999; Kuruvilla et al., 2000; Nishimune et al., 2000). This results in the recruitment and activation of a number of serine/threonine kinases, including Akt. In neurons, the role of Akt in survival is well established (Crowder and Freeman, 1998; Dudek et al., 1997; Eves et al., 1998; Philpott et al., 1997), however that of PI3K is more controversial. There have been several reports that inactivation of PI3K interferes with NGF-induced neuronal survival, particularly in sympathetic neurons (Crowder and Freeman, 1998; Hetman et al., 1999; Kuruvilla et al., 2000; Mazzoni et al., 1999;

Vaillant et al., 1999; Yao and Cooper, 1995). However, other studies suggest that PI3K has only a modest role in survival (Philpott et al., 1997; Tsui-Pierchala et al., 2000; Virdee et al., 1999). One possible explanation for these ambiguous results is that the approach most commonly taken in such studies is the use of dominant negative constructs. This approach, unfortunately, also runs the risk of interfering with the function of other proteins downstream of the PI3K, several of which may themselves be implicated in regulating cell survival (Brunet et al., 2001). Alternatively, certain neuronal populations may require cooperation with other intracellular signalling pathways to promote the full survival effect of a particular neurotrophic factor (Datta et al., 2000; Lizcano et al., 2000; Pierchala et al., 2004; Shaywitz and Greenberg, 1999; Virdee et al., 2000)

The consequences of Akt phosphorylation are varied. Akt is capable of regulating transcription factors of several classes, for example members of the Forkhead family, NFκB and cAMP-responsive element binding protein (CREB) (Biggs, III et al., 1999; Brunet et al., 1999; Du and Montminy, 1998; Gustin et al., 2004; Kane et al., 1999; Kane et al., 2002; Kops et al., 1999; Kops and Burgering, 1999; Ozes et al., 1999; Romashkova and Makarov, 1999). The forkhead family of transcription factors regulates the expression of pro-apoptotic proteins, such as FasL, and phosphorylation of these factors by Akt inhibits their function (Biggs, III et al., 1999; Brunet et al., 1999; Kops and Burgering, 1999). In contrast, NFκB and CREB are activated through Akt phosphorylation and can cause the transcription of anti-apoptotic proteins such as Bcl-xL and Bcl-2 (Chen et al., 2000; Riccio et al., 1999) as well as additional neurotrophin expression (Tao et al., 1998). Akt can also directly manipulate the apoptotic machinery, for example by phosphorylating Bad, thus preventing it from inactivating both Bcl2 and Bcl-xL (Datta et al., 1997; Del Peso et al., 1997; Zha et al., 1996) and may act to indirectly promote cell survival by regulating cellular metabolism (Brunet et al., 2001).

Cytokines commonly activate members of the signal transducer and activator of transcription (STAT) family of transcription factors to promote cell survival (Hirano et al., 2000). In the nervous system, STAT-3 has been reported to mediate CNTF-induced survival in nodose neurons (Alonzi et al., 2001). Indeed Nishimune et al

(2000) proposed the STAT-3 and PI3K/Akt signalling were inextricably linked in promoting CNTF-induced motoneuron survival, where STAT-3 induction by cytokines led to the production of Reg-2. In these neurons, Reg-2 subsequently acts as an obligatory autocrine or paracrine trophic factor via PI3K and Akt. Akt activation by cytokines is also dependent on functional STAT-3 in nodose neurons (Alonzi et al., 2001). In this case Akt appears to promote survival through the activation of NFκB as interference with NFκB signalling also prevents CNTF-induced neuronal survival (Middleton et al., 2000; Nishimune et al., 2000). STAT-3 may also function by directly manipulating the apoptotic machinery as it is capable of inducing anti-apoptotic proteins BAX and Bcl-xL in lymphocyte and myeloid and fibroblast cell lines respectively (Catlett-Falcone et al., 1999; Fukada et al., 1996; Karni et al., 1999), thus mediating survival induced by granulocyte colony stimulating factor (G-CSF) in the myeloid cell line (de Koning et al., 2000)and by interleukin-6 (IL-6) in lymphocyte and hepatoma cell lines (Chen et al., 1999; Fukada et al., 1996).

Three MAPK cascades, converging on extracellular-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK, have been implicated in regulating cell survival in the nervous system. The ERK cascade is generally associated with cell survival promoted by neurotrophic factors, while the JNK and p38 cascades are mainly activated in situations involving external stresses such as radiation or pro-inflammatory cytokines (Tibbles and Woodgett, 1999). Activation of the ERK cascade is anti-apoptotic, while JNK and p38 are pro-apoptotic, with mice lacking JNK genes showing reduced neuronal apoptosis to noxious stimuli and during development (Cheung and Slack, 2004; Harper and LoGrasso, 2001; Kuan et al., 1999). The classical ERK cascade begins with the recruitment of the low molecular weight G-protein Ras to receptors such as Trks. This results in the sequential activation of Raf, MEK1/2, ERK1/2 and RSK (ribosomal S6 kinase). RSK can then perform a number of functions, including inhibiting the pro-apoptotic protein BAD or activating CREB (Bonni et al., 1999; Finkbeiner, 2000; Shimamura et al., 2000). MEK/ERK, in some case though CREB, can regulate the transcription of a number of anti-apoptotic genes including Bcl-2, Bcl-xL, Mcl-1 and those of survival factors

such as BDNF itself (Ballif and Blenis, 2001; Boucher et al., 2000; Huang et al., 2000a; Jost et al., 2001; Leu et al., 2000; Pugazhenthi et al., 1999; Riccio et al., 1999; Tao et al., 1998). NGF-dependent survival of sympathetic neurons has been shown to be partially dependent on CREB (Riccio et al., 1999). In the peripheral nervous system, the importance of ERK signalling in neurotrophic factor-induced neuronal survival varies. Neuronal survival through TrkB has been found to be partially dependent on MEK (Atwal et al., 2000), however NGF-induced neuronal survival in sympathetic and DRG neurons is independent of ERK1/2 (Creedon et al., 1996; Klesse and Parada, 1998; Virdee and Tolkovsky, 1996). Several points of crosstalk between PI3K and ERK signalling cascades have been identified. PI3K can both negatively and positively regulating the activities of Ras, Raf and MEK, depending on the signalling context and 3'-phosphoinositide-dependent protein kinase (PDK), and Akt, kinases downstream of PI3K can activate RSK and CREB respectively (Finkbeiner, 2000; Guan et al., 2000; King et al., 1997; Rommel et al., 1999; Wennstrom and Downward, 1999; York et al., 2000; Zimmermann and Moelling, 1999) and in DRG neurons, York et al. (2000) showed that ERK activation by NGF requires PI3K.

1.5.3. Intracellular signalling and regulation of neurite outgrowth by neurotrophic factors in the developing peripheral nervous system

Several different intracellular signalling pathways and second messenger systems have been implicated in regulating axonal growth and the establishment of innervation in the nervous system. As axonal outgrowth is typically regulated by neurotrophic factors, electrical activity and extracellular matrix proteins, the pathways studied with respect to neurite outgrowth are pathways typical of those activated by such stimuli. These pathways include calcium- and cyclic nucleotide-based pathways, as well as those using protein kinases and phosphatases (Keith and Wilson, 2001). Again, in this section I will discuss only the signalling pathways relevant to neurotrophic factor-induced neurite outgrowth.

One of the most widely studied pathways implicated in neurotrophic factor-induced neurite outgrowth is the Ras-activated Raf-Mek-Erk cascade. NGF, BDNF and NT-4

can activate the ERK pathway in a range of embryonic and adult neurons and in some cases this activation is localised to the axonal compartment, and not the cell body (Becker et al., 1998; Cowley et al., 1994; Howe et al., 2001; Minichiello et al., 1998; Sjogreen et al., 2000; Susen et al., 1999; Svensson and Ekstrom, 1998). In the peripheral nervous system, sympathetic neurons have been shown to require functional ERK signalling, as well as PI3K or calcium signalling pathways in order to elicit normal neurite outgrowth in response to BDNF or NGF respectively (Atwal et al., 2000; Svensson and Ekstrom, 1998; Vaillant et al., 2002) and sensory neurons of the DRG require Ras, Raf and Mek, as well as PI3K for NGF-induced axonal growth (Liu and Snider, 2001; Markus et al., 2002a; Markus et al., 2002b; Sjogreen et al., 2000) Outgrowth in adult nodose neurons, induced by NT-4/5, is also dependent on MEK activation, as well as PKA (Wiklund and Ekstrom, 2000). Activation of this MAP kinase pathway alone may be sufficient to support axonal growth in some neurons. Gain of function studies in PC12 cells, as well as in bax null DRG neurons, show that transfection with activated forms of Ras, C-Raf or Erk2 in the absence of trophic support is capable of inducing robust neurite extension equivalent to that of NGF (Markus et al., 2002b; Robinson et al., 1998). Targets of Erk1 and Erk2 that may mediate effects on neurite outgrowth include MAP2 and tau, which have also been shown to be downstream targets of neurotrophins (Vaillant et al., 2002). Phosphorylation of MAP2 induces increased association with, and consequently stabilisation of, microtubules (Brugg and Matus, 1991; Jameson and Caplow, 1981; Sanchez et al., 2000a; Vaillant et al., 2002).

PI3K has also been implicated in neurite outgrowth in neurons stimulated by neurotrophins (Liu and Snider, 2001). One mechanism through which PI3K could affect neurite outgrowth is by regulating the activation of Erk via Ras and Rap1 (York et al., 2000). The downstream target of PI3K, Akt, has also been implicated in regulating axon calibre and branching in NGF-stimulated DRG neurons (Markus et al., 2002b). Indeed in adult DRG neurons, NGF and insulin-like growth factor-1 (IGF-1) act in a synergistic manner to promote neurite outgrowth through PI3K, Akt and GSK-3, a pathway that was found to be more important in regulating neurite outgrowth than MAP kinase signalling (Jones et al., 2003). GSK-3 can phosphorylate

MAP2C and consequently regulate the stability of microtubules (Sanchez et al., 2000b). PI3K could also activate Rac1. Rac proteins have a number of targets that can modulate the cytoskeleton (Lundquist, 2003; Miller and Kaplan, 2003; Yasui et al., 2001). Pak1 stimulates LIM kinase activity, which can then inhibit cofilin, an actin-depolymerising protein to modulate dendritic growth (Lundquist, 2003). Another downstream target of Rac1, JNK1 has also been shown to be important in maintaining dendritic microtubules (Chang et al., 2003).

Calcium-dependent signalling pathways are predominantly involved in mediating activity-induced neurite outgrowth (Lohmann et al., 2002; Miller and Kaplan, 2003; Redmond et al., 2002; Vaillant et al., 2002). Calcium-activated proteins such as calcium/calmodulin dependent kinase (CAMKII) phosphorylate MAP2 thus causing stabilisation of dendritic microtubules (Sanchez et al., 2000b; Sanchez et al., 2000a; Vaillant et al., 2002; Wu and Cline, 1998). Interestingly, CAMKII has also been reported to limit axonal and dendritic arbour elaboration during the maturation process (Zou and Cline, 1999). Most recently Fink et al. (2003) reported that it is the CaMKIIβ isoform that can regulate dendritic morphology in hippocampal neurons and Gaudilliere et al. (2004) reported that CaMKII regulates activity-dependent changes in dendritic morphology in cerebellar granule cells through the transcription factor NeuroD. Activity-dependent changes in calcium levels can also act to promote neurite outgrowth through the CaMKIV-CREB pathway (Redmond et al., 2002). Activity-dependent outgrowth also requires the Ras-MEK-Erk pathway, and it has been proposed that neurotrophin- and activity-dependent signalling of dendritic outgrowth converge on both the Ras-MEK-ERK- and calcium-dependent signalling pathways (Vaillant et al., 2002). Neurotrophins activate both CaMKII and CaMKIV through PLCγ activation, which stimulates calcium ion release from intracellular stores and are implicated in neurotrophin-induced neurite outgrowth in some circumstances (He et al., 2000; Kaplan and Miller, 2000; Minichiello et al., 2002; Vaillant et al., 2002). Neurotrophins can also directly initiate membrane depolarisation through coupling with sodium channels (Blum et al., 2002; Kovalchuk et al., 2002).

Several additional proteins have been implicated in regulating neurite outgrowth in certain circumstances. Neurite outgrowth in adult nodose neurons stimulated by NT-4/5 can also be inhibited by blocking PKA or PKC signalling (Wiklund and Ekstrom, 2000) and PKC has also been implicated in axonal growth and neurite initiation in hippocampal neurons (Cabell and Audesirk, 1993), although NGF-stimulated outgrowth in embryonic DRG neurons is not affected by inhibition of PKC (Liu and Snider, 2001).

Novel interactions between neurotrophin receptors and other intracellular components that regulate neurite outgrowth are being discovered. Recently the p75 neurotrophin receptor has been found to interact directly with the RhoA protein (Yamashita et al., 1999). RhoA is a member of the Ras family of GTP-binding proteins that is known to regulate the organisation of the actin cytoskeleton in a range of cell types (Mackay and Hall, 1998). The actions of Rho are thought to oppose the actions of Rac, mentioned earlier in the context of PI3K, and cdc42, two proteins that positively regulate actin-dependent growth(Etienne-Manneville and Hall, 2002). p75 constitutively activates RhoA by acting as a displacement factor, releasing RhoA from a complex with Rho-GDP dissociation inhibitor (Rho-GDI) (Yamashita and Tohyama, 2003). Inactivating RhoA, by overexpressing a RhoA inactivating protein C3 transferase or a dominant negative construct enhanced neurite outgrowth whereas overexpression of a constitutively active RhoA blocks neurotrophin-induced neurite growth in trigeminal or ciliary neurons (Davies, 2000b; Ozdinler and Erzurumlu, 2001; Yamashita et al., 1999). Neurotrophin binding to p75 blocks constitutive RhoA activation (Yamashita et al., 1999). RhoA mediation of neuritic growth depends on Rho-associated kinase (ROK), as ROK inhibition blocks the actions of constitutively active RhoA in hippocampal neurons and activated ROK also limits dendritic length (Nakayama et al., 2000). ROK is thought to act by controlling the phosphorylation of myosin light chains and actomyosin contractility (Hirose et al., 1998; Kimura et al., 1996; Winter et al., 2001). RhoA and ROK also mediate the effects of electrical activity withdrawal on neurite outgrowth (Nakayama et al., 2000; Sin et al., 2002). In that situation, ROK has been reported to act through lim kinase (LIMK) which can activate cofilin, an actin depolymerisation factor

(Etienne-Manneville and Hall, 2002; Meng et al., 2002; Meyer and Feldman, 2002). As RhoA has been implicated in dendritic, rather than axonal, development in a number of neuronal types this might prove to be a mechanism through which neurotrophins influence dendritic rather than axonal morphology (Lee et al., 2000; Li et al., 2000; Nakayama et al., 2000; Ruchhoeft et al., 1999; Wong et al., 2000).

So far I have principally described the ways in which trophic factor stimulation, particularly using neurotrophins, can influence cytoskeletal dynamics to alter normal axonal and dendritic growth in a transcription independent manner. Changes in gene transcription through specific transcription factor activation have also been implicated in affecting neurite outgrowth. CREB is one such factor. CREB can be activated through Ras/ERK signalling and in sympathetic and sensory neurons is phosphorylated in response to NGF exposure (Lonze and Ginty, 2002; Xing et al., 1996). Although sensory neurons undergo apoptosis by E17.5 in $CREB^{-/-}$ mice, at E13.5 a normal number complement of neurons are present and early axonal defects are seen. Similar axonal defects are seen at a later age in the $CREB^{-/-}/Bax^{-/-}$ mutant, in which neuronal survival is rescued (Lonze et al., 2002). *In vitro*, dominant-negative CREB constructs also inhibit neurite outgrowth of cortical neurons (Redmond et al., 2002). Axon targeting in DRG neurons in response to NT-3 has also been shown to be transcription dependent. Expression of transcription factor ER81 in these neurons is dependent on NT-3 and its expression abolished if the target field is removed from the developing embryo (Lin et al., 1998a). In mice lacking Er81, the central projection of DRG neurons does not extend further than the intermediate zone (Arber et al., 2000). Finally, as mentioned earlier, CaMKII activates NeuroD to mediate its actions on neurite outgrowth in cerebellar granule cells (Gaudilliere et al., 2004).

Chapter 2

NFκB signalling regulates neurite outgrowth in peripheral sensory neurons

2.1. Introduction

Nuclear factor κB (NFκB) is a ubiquitously expressed transcription factor that regulates the expression of many different proteins involved in a variety of cellular processes, including innate and adaptive immune responses, stress responses, cell survival, proliferation and differentiation (Baldwin, Jr., 1996; Karin and Lin, 2002; Li and Stark, 2002). It consists of homo- and heterodimers of any of p65 (Rel A), RelB, c-Rel, p50 or p52 proteins, although it is the p50/p65 heterodimer that is predominant in most cells types, and which is commonly referred to simply as NFκB (Karin and Lin, 2002; Li and Verma, 2002). Active NFκB dimers are detected principally in the nucleus, while, in the majority of cell types, the basal condition is for these dimers to be held inactive in the cytosol through their interaction with inhibitory proteins of the IκB family. This family consists of 7 family members: IκBα, IκBβ, IκBε, IκBγ, Bcl-3, as well as p100 and p105, the precursor proteins for p52 and p50 respectively (Hayden and Ghosh, 2004; Yamamoto and Gaynor, 2004). It is the degradation of these inhibitory proteins by the proteosome, induced by their phosphorylation and subsequent ubiquitination and usually occurring as a consequence of the binding of an extracellular signalling molecule, that reveals the NFκB nuclear localisation sequence and allows its translocation to the nucleus. The degradation of these inhibitory proteins also reveals phosphorylation sites whose state can influence the transcriptional activation ability of NFκB (Li and Stark, 2002; Sizemore et al., 1999; Wang et al., 2000; Zhong et al., 1997; Zhong et al., 1998). Once located in the nucleus, NFκB binds to the promoter and enhancer regions of genes containing κB consensus sequences, thus influencing gene transcription (Baldwin, Jr., 1996; Karin, 1999).

NFκB expression is found throughout the central and peripheral nervous systems, in both neuronal and non-neuronal cells (Bhakar et al., 2002; Maggirwar et al., 1998; Nickols et al., 2003; O'Neill and Kaltschmidt, 1997). The ways in which NFκB influences cells in the nervous system are still being defined, with NFκB being implicated in the signalling pathways of a growing number of extracellular signals, including neurotrophic factors, cytokines and neurotransmitters (Carter et al., 1996a; Digicaylioglu and Lipton, 2001; Guerrini et al., 1995; Hamanoue et al., 1999; Kaltschmidt et al., 1995; Nishimune et al., 2000; Yalcin et al., 2003). NFκB has been found to promote the neuronal survival both during development (Bhakar et al., 2002; Hamanoue et al., 1999; Maggirwar et al., 1998) and after a wide range of toxic insults (Barger et al., 1995; Daily et al., 2001; Digicaylioglu and Lipton, 2001; Fridmacher et al., 2003; Lipsky et al., 2001; Mattson et al., 2000; Piccioli et al., 2001; Tamatani et al., 2000; Yu et al., 2000). In some circumstances, however, it has also been implicated in promoting neuronal death (Pizzi et al., 2002; Post et al., 1998; Schneider et al., 1999; Shou et al., 2002; Uberti et al., 2000). In cerebellar granule cells and hippocampal neurons, at least, this difference has been attributed to activation of different NFκB subunits, and manipulation of the expression of these subunits has been sufficient to convert positive effects on survival into negative ones (Pizzi et al., 2002). NFκB is also involved in the adaptations that occur in response to inflammatory conditions and neurodegenerative disease (Bhakar et al., 2002; Blondeau et al., 2001; Clemens et al., 1997; Fridmacher et al., 2003; Gabriel et al., 1999; Gentry et al., 2000; Grilli and Memo, 1999; Mattson et al., 2000) and in non-neuronal cells NFκB also participates in peripheral nerve myelination (Nickols et al., 2003).

An additional role for NFκB in processes related to learning and memory has recently emerged. N-methyl-d-aspartate receptor activation by glutamate, in hippocampal neurons and cerebellar granule cells stimulates nuclear translocation of NFκB (Guerrini et al., 1995; Scholzke et al., 2003), and interruption of NFκB-induced transcriptional activation, using κB decoy DNA, interferes with both long-term potentiation and long-term depression in hippocampal slice cultures (Albensi and Mattson, 2000). That these changes are physiologically relevant is indicated by

interference with NFκB, either pharmacologically or through transgenic technology, preventing memory formation in the crab, interfering with spatial learning in mice and inhibiting fear conditioning in rats (Meffert et al., 2003; Merlo et al., 2002; Yeh et al., 2002). One important physiological alteration that is associated with the adaptive changes involved in learning and memory is synaptic remodelling, where certain terminal branches extend and retract as a consequence of inputs to the neurons from a combination of synaptic activity and retrograde signalling by either neurotransmitters or neurotrophic factors (Balderas et al., 2004; Tyler et al., 2002; Vicario-Abejon et al., 2002). This process is thought to be mediated by events that, to a certain extent, reiterate the processes that determine neuronal morphology during development (Arendt, 2003; Keith and Wilson, 2001; Lang et al., 2004; Reuss and Halbach, 2003; Zhou et al., 2004b). This prompted us to question whether NFκB could also be involved in neuronal process growth, elaboration and modification during development.

In order to investigate the role of NFκB in neurite outgrowth during development I studied neurite outgrowth from neurons of the nodose ganglion. The nodose ganglion is a well-defined population of peripheral neurons that have been well characterised both in vitro and in vivo, and cultures of these neurons can be easily established and maintained in vitro in the presence of BDNF (Davies et al., 1993). Unlike other populations of peripheral neurons, such as those of the SCG or trigeminal ganglion, no link has been described between NFκB signalling and survival in these neurons (Hamanoue et al., 1999; Maggirwar et al., 1998), a dissociation confirmed by the experiments shown in this chapter, making them ideal for studying outgrowth without the confounding effects of changes in neuronal survival. These studies show that preventing NFκB activation by interfering with IκBα degradation or inhibiting the transcriptional activity of NFκB reduces both the overall length and the complexity of nodose neurons. This effect is limited to a distinct developmental window between E18 and P1.

2.2. Materials and Methods

2.2.1. Neuronal culture

Dissociated cultures of nodose ganglion neurons were set up from embryos and early postnatal mice obtained from overnight matings of CD1 mice. The dissected ganglia were trypsinized and dissociated by trituration and the neurons plated in defined serum-free medium, on a polyornithine/laminin substratum, in 35mm tissue culture dishes (Davies et al., 1993).

The murine nodose ganglia are situated just below the base of the skull, close to the jugular foramen (see fig 2.1). Embryonic mice were removed, following cervical dislocation of the pregnant mother, decapitated and the heads collected in Leibowitz-15 medium (L-15) without sodium bicarbonate. Postnatal mice were killed by carbon dioxide asphyxiation, followed by decapitation. The top of the skull and the forebrain were removed and the head was bisected in the sagittal plane. The jugular foramen was then exposed by removal of the hindbrain and opened as shown in figure 2.1, revealing the nodose and superior cervical sympathetic (SCG) ganglia underneath. The nodose ganglion is a distinctive spherical structure with a prominent vagus nerve coming from is distal aspect. The SCG is elongated and lies lower down, attached to the thinner sympathetic chain. The nodose ganglion was removed from the head by grasping the base of the vagus nerve, and then cleaned of all nerve and connective tissue using tungsten needles. All dissections were performed in a laminar flow hood, using a stereomicroscope and a fibre-optic light source to prevent overheating of the tissue. Dissection instruments were sterilised by flaming in alcohol. Tungsten needles were produced by electrolytically sharpening tungsten wire (0.5mm diameter) in 1M KOH. The needles were held in chuck-grip platinum wire holders (Davies, 1988).

Dissected ganglia were trypsinized by incubation in 0.1% trypsin in calcium and magnesium free Hanks balanced salt solution (CMF-HBSS; GibcoBRL) for between 15 and 25 minutes at 37°C, washed in Ham's F12 medium (GibcoBRL) supplemented with 10% heat-inactivated horse serum (HIHS; GibcoBRL) so as to

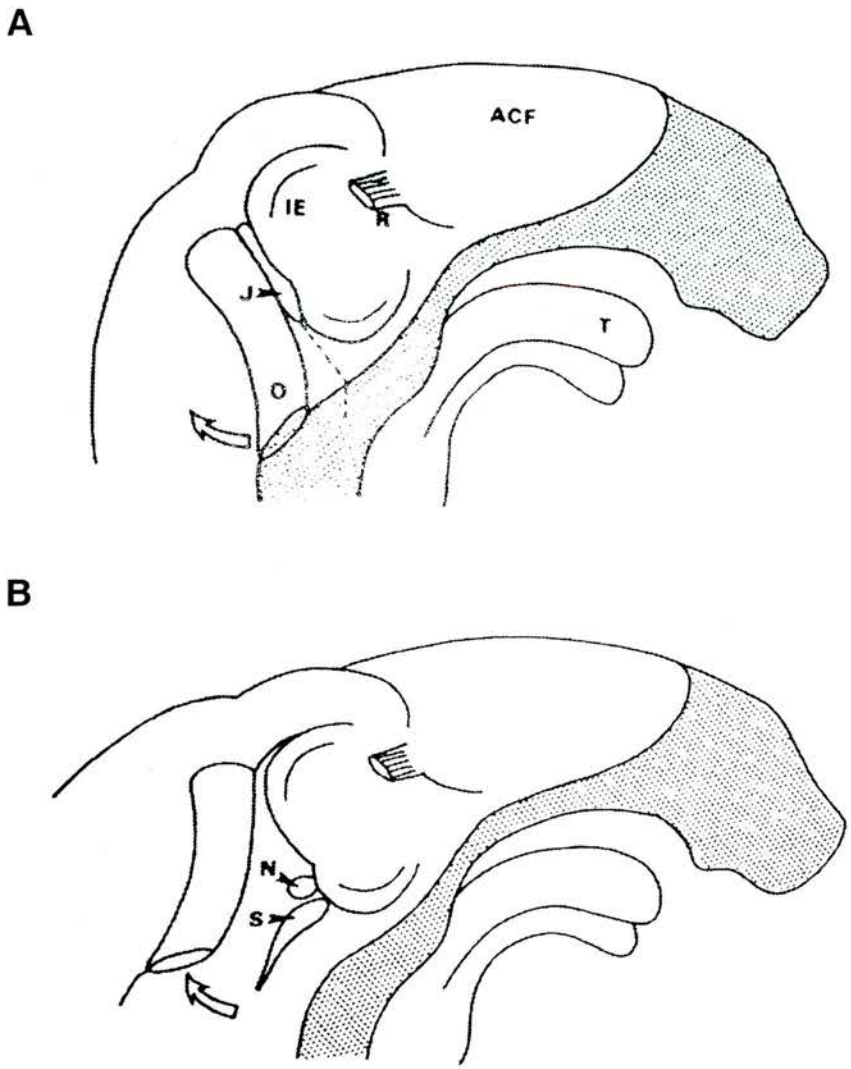

Figure 2.1 *Dissection of the nodose ganglia*
This diagram shows the medial aspect of half of the head of an E14 mouse embryo at two stages of dissection. The shaded area represents the bisected midline structures. A) An incision is made from the jugular foramen (J) to the midline is made (dotted line) and the occipital bone reflected in the direction of the arrow to open the jugular foramen. B) the nodose (N) and superior cervical ganglion (S) are revealed underneath. (T- tongue, ACF-anterior cranial fossa, IE-inner ear, R-root of the trigeminal nerve) From Davies, 1995.

stop trypsin activity. The ganglia were transferred into defined, serum-free medium (F-14 medium supplemented with 2mM L-glutamine and SATO supplement (composition in appendix) and dissociated by trituration using a fire-polished siliconized Pasteur pipette. The neurons were plated on 35mm tissue culture dishes prepared by overnight incubation with polyornithine (0.5mg/ml poly-DL-ornithine hydrobromide, Sigma in 0.15M borate buffer, pH 8.4) at room temperature, the dishes were washed, dried and then incubated for 3 hours with laminin (20μg/ml in CMF-HBSS, Sigma) at 37°C.

Different density cultures were required for different experiments. Ballistic transfection requires an initially high neuronal density in order to achieve transfection of sufficient numbers of neurons, whereas the cultures established from transgenic mice or for use with pharmacological inhibitors need to be low density so that the neuritic arbour of individual neurons can be distinguished.

If the culture was destined for ballistic transfection, the substratum was prepared by placing 50μl of laminin in a droplet in the centre of the dish. Neurons were then plated by replacing the laminin with a 50μl droplet of the cell suspension in supplemented F14 (as described earlier) containing 1000-3000 neurons. For cultures that were to be treated with pharmacological inhibitors, 80μl of laminin was added to the centre of the dish and spread over a circular area covering three quarters of the 35mm dish. 1ml of cell suspension was then plated directly into 1ml of supplemented F-14 medium to give a final density of 500-1000 neurons per dish. In both cases, the dishes were incubated at 37.5°C in a humidified 3.5% CO_2 incubator for 3-4 hours to allow the cells to attach. Neurotrophic factor (BDNF, R and D, 10ng/ml) was added after either ballistic transfection or an initial cell count followed by a pre-incubation of 45 minutes with the relevant pharmacological inhibitor.

2.2.2. Genotyping transgenic mice

IKK-α knockout embryos were generated by mating mice heterozygous for null mutations in the IKK-α gene (Hu et al., 1999; Takeda et al., 1999). Pregnant females were killed at embryonic day 18 just before the age at which embryos were found to die *in utero*, and tissue samples for genotyping collected from each embryo

immediately prior to dissection of the ganglia of interest. DNA was extracted using the FastDNA kit (Qbiogene, UK) following the manufacturers instructions. Briefly, the tissue was homogenised and the cells were lysed in the supplied lysing matrix and buffer. The supernatant was removed and incubated with DNA binding matrix, which was then washed using an ethanol-based solution and the DNA eluted using ultra-pure water. Polymerase chain reaction (PCR) was used to ascertain the genotype of each embryo. For IKKα null, heterozygous and wildtype mice, four primers were used in the amplification, giving a 400bp product for the wildtype allele (5'-ACA ATA CAT AAA ATA ATT GAT-3' and 5'-TGA AAT GTT TCC ACT ACC ATT-3') and a 600bp product for the null allele (5'-CAA CAT TAA ATC TCA GCG AG-3' and 5'-GGA ACA TTG CAG TAT TTG G-3'), which were separated on a 2% agarose gel and visualised using ethidium bromide.

The PCR reaction was prepared using a reaction master mix of a volume appropriate to the number of embryos (see appendix for composition). For each embryo the PCR reaction was carried out in duplicate and positive (known heterozygote DNA) and negative (DNA-free) control used. Each reaction was performed in a 20μl reaction volume, in a 500μl microfuge tube and layered with mineral oil to prevent evaporation during cycling. An initial denaturation step of 15mins at 95°C was followed by 35 cycles consisting of a 40 second denaturation at 95°C, primer annealing at 55°C for 1min and extension at 72°C for 1min. An additional final extension phase of 10mins at 72°C was used.

2.2.3. Ballistic transfection

Particle mediated gene transfer, or ballistic transfection, has been shown to be an effective way of transferring genes into neurons (Gutierrez et al., 2004; Sole et al., 2004). Compared to other methods of gene transfer, such as viral vectors, chemical methods, such as lipofection, or microinjection, it is quick, non-toxic and causes no interference with global gene expression. The process of ballistic transfection consists of three steps (Figure 2.2A). First the DNA is coated onto inert microcarriers, and then these particles transferred onto a macrocarrier, such as Teflon tubing, for loading into the gene gun. The final step is to inject the target cells with

the microcarriers using a motive force such as high-pressure helium. In the experiments reported in this thesis, a hand-held gene gun (Helios Gene-Gun, Byroad, Hercules, CA USA) was used in conjunction with a gold microcarrier as described in (Gutierrez et al., 2004).

2.2.3.1. Preparation of gold particle cartridges

Gold particles were prepared using the manufacturers protocol. 20mg of 1.6μm gold particles were suspended in 100μl of spermidine (50mM) plus 20μg of pYFP (Clontech) and either the plasmid of interest, such as pSR-IκB-α, or a pCDNA control plasmid for 15 minutes. The DNA-coated gold particles were precipitated with 100μl of 2M $CaCl_2$, washed with 100% ethanol (3 washes) and resuspended in 1.2 ml of 100% ethanol plus 0.01 mg/ml polyvinylpirrolidone. The interior wall of Teflon tubing was coated with this mixture. The tubing was dried using nitrogen gas and cut into cartridges.

κB decoy DNA was prepared by annealing complimentary single-stranded oligonucleotides (5'-GAGGGGACTTTCCCT-3' and 5'-AGGGAAAGTCCCCTC-3') containing a κ-B DNA binding sequence previously been described as effectively knocking down NFκB dependent gene expression in neurons (Yeh et al., 2002). Control DNA with a scrambled sequence was also prepared by annealing oligonucleotides of the following sequence: 5'-GATGCGTCTGTCGCA-3' and 5'-TGCGACAGACGCACT-3'. Double stranded DNA solutions were prepared at 50mM, and were ethanol precipitated onto the gold particles in addition to the pYFP marker plasmid.

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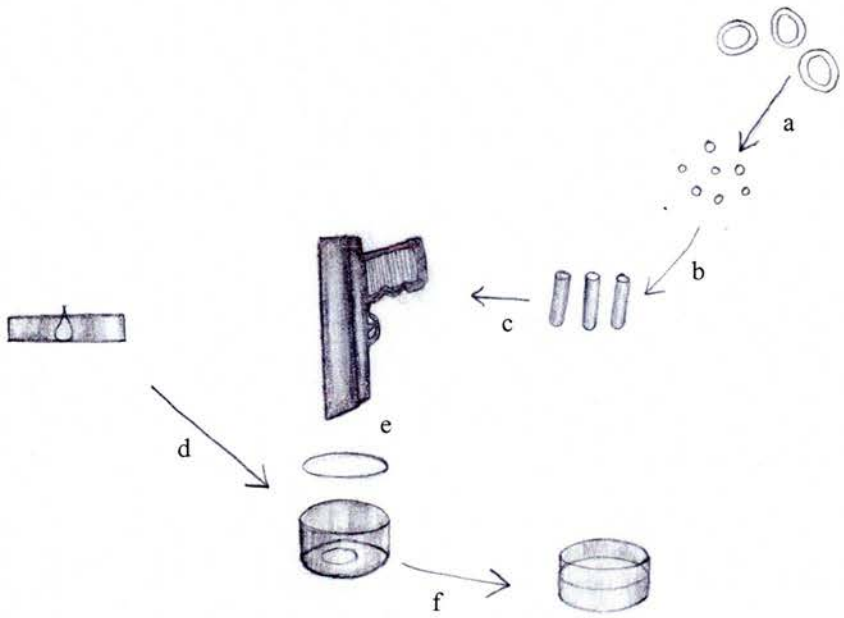
* **Figure 2.2** *Ballistic transfection*
A) Schematic diagram showing the process of ballistic transfection. Plasmid DNA is coated onto inert microcarriers (1.6μm gold particles; a) that are subsequently loaded onto a macrocarrier (teflon tubing; b) for insertion into the gene gun (c). Culture medium is then removed from the previously prepared culture of nodose neurons (d) and the DNA-coated microcarriers transferred into the cells using high pressure helium as a motive force (e). Culture medium is the replaced, this time containing the required trophic factors (f). B) Photomicrographs showing neonatal neurons expressing pYFP as a result of ballistic transfection

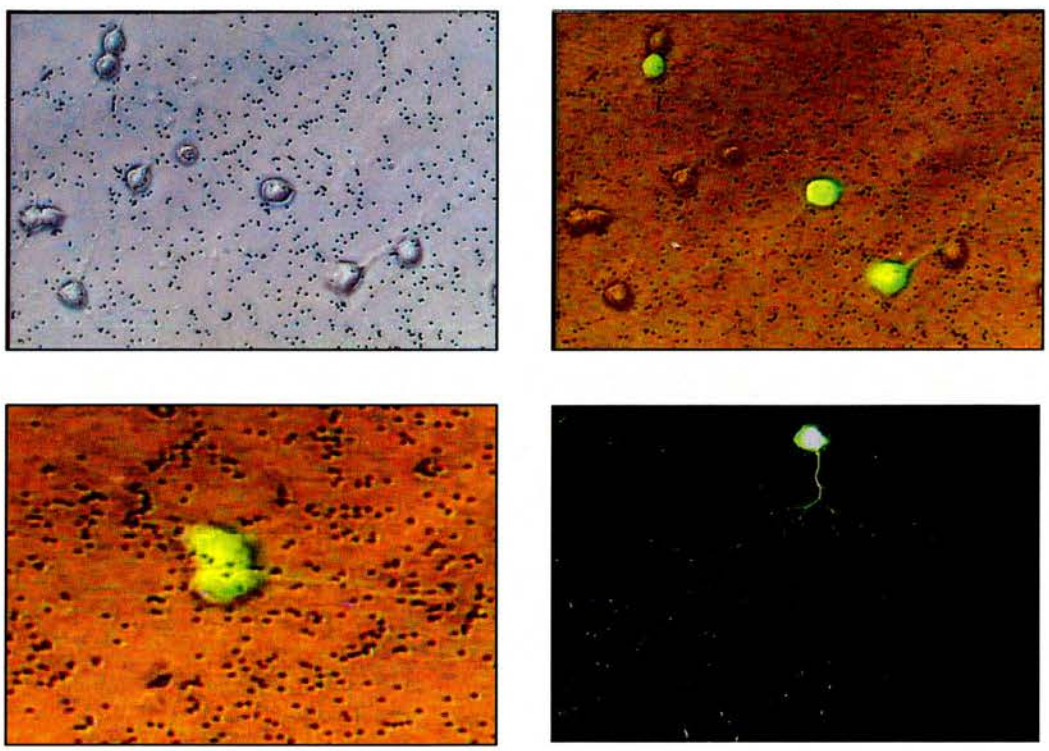
A



B

Normal light

Filtered (488) and low intensity normal light



Filtered (488) and low intensity normal light

Filtered (488) light

Figure 2.2

2.2.3.2. Neuronal transfection using the gene-gun

Neurons were transfected using the gene-gun three hours after plating, by which time the cells were securely attached to the dish. Immediately prior to transfection the culture medium was removed from the dish and the gold particles were shot into the cultured neurons with the gun pressurized at 140psi. The gun was held approximately 3cm above the dish, and a 70μm nylon mesh screen placed between the gun and the culture to prevent the cells being damaged by the shock wave. After transfection 2ml of supplemented F14 with or without 10ng/ml BDNF were added to each dish.

Figure 2.2B shows a culture transfected with a YFP reporter plasmid (pYFP, Clontech), visualised using both bright field and filtered light. Neuron cell bodies and gold particles (small black dots) can be seen by bright field optics (top left). The top right panel shows the same field at lower intensity bright field, superimposed with the YFP fluorescent image. Another pair of cells is shown in the bottom left panel. The bottom right panel shows a single YFP positive neuron under filtered light alone, where the neuritic arbour can be seen in addition to the cell body.

In addition to the YFP (pYFP, Clontech) and RFP (pDSred2, Clontech) reporter plasmid the following plasmids were used: Super-repressor IκBα expression plasmid (pCDNAIII, S32A/S36A mutation, Rodriguez *et al.*, 1996), p65 expression plasmid (pCDNAIII, human wild-type p65, Rodriguez *et al.*, 1996), NIK wildtype (pCDNAIII, human, gifted by R.T.Hay, St. Andrews University) and mutant expression plasmids (pCDNAIII, K429A/K430A mutation, gifted by R.T.Hay, St. Andrews University), and an p50-p65 promoter-controlled GFP expression plasmid (Gifted by J. Comella, University of Llieda).

2.2.4. Quantification of fluorescence

Quantification of neuronal fluorescence was performed to estimate the relative level of NFκB activation in neurons transfected with a plasmid expressing GFP under the control of an NFκB promoter. Neurons were transfected with this plasmid along with pRFP, to allow the identification of transfected cells, incubated for 24h with or without BDNF (20ng/ml) and subsequently imaged with a Zeiss Axioplan laser

scanning confocal microscope. The mean intensity of fluorescence of each cell body was obtained using the LSM510 software and the standard 255 intensity scale after subtraction of the background intensity. In each experimental condition all imaging and quantification was performed blind. 40-60 neurons were assessed per condition and statistical comparisons were performed using ANOVA followed by Fishers' post-hoc test. The results shown are from a single experiment that is representative of the results seen in three separate experiments. In all experiments reporter gene expression was also analysed ratiometrically, that is by expressing the level of GFP fluorescence of each cell relative to the level of RFP fluoresence. In all cases this analysis showed the same changes as those seen with GFP fluorescence alone, and is therefore not presented.

2.2.5. Estimating neuronal survival

Estimates of neuronal survival in cultures treated with pharmacological inhibitors were obtained by counting the number of neurons surviving after 24 hours in culture using phase-contrast microscopy, as compared to an initial count just prior to addition of the relevant inhibitor. Two hours after plating the number of neurons present in a 12 x 12mm grid in the centre of the dish was counted. Immediately after this count, the pharmacological inhibitor or control compound was added to the culture medium, followed, after 45 min, by the addition of BDNF (10 ng/ml) to half the dishes in each condition. A second count was performed 24h after the addition of inhibitor, directly before the cells were fixed for immunocytochemistry. The number of neurons surviving at 24h is expressed as a percentage of the initial number of neurons.

Estimates of neuronal survival in cultures transfected using the gene gun were performed differently. These were obtained by counting the number of YFP-labelled neurons surviving 48 hours after transfection. This count was then expressed as a percentage of the number of labelled neurons counted 12 hours after transfection, the earliest time point at which all the cells expressing YFP can be reliably seen. Each cell was also examined using phase-contrast conditions to corroborate cell survival. The area counted was defined by the area of each culture dish in which gold particles

were seen to be embedded. Despite the initial neuronal count in this method being carried at 12 hours after transfection, this method of quantifying neuronal survival is unlikely to miss the death of significant numbers of nodose neurons, as this particular population of neurons, unlike neurons of the superior cervical ganglia, have a slow rate of death even in the absence of trophic factors (26% death at 12h, compared to 97% death at 12h in SCG). In all cultures, this has been confirmed by examining the absolute counts of transfected neurons after 12 and 48 hours, and none of which reveal significant differences in absolute cell numbers in BDNF-treated transfected and untransfected neurons.

For the survival data presented in figure 2.7, the percentage survival for each dish is then expressed as a percentage of the mean survival of the BDNF-treated control-transfected group, and statistical comparisons performed using ANOVA followed by Fishers' post-hoc test. Triplicate cultures were set up for all conditions and the data shown is compiled from 2-4 separate experiments for each age.

2.2.6. Analysis of neurite outgrowth

Analysis of neurite outgrowth was performed on images acquired digitally using an Axioplan Zeiss laser-scanning confocal microscope. Images of transfected, YFP-labelled neurons were of live neurons that could be visualized directly, while cultures treated with pharmacological inhibitors were first fixed and fluorescently labelled for the neuronal-specific marker β-III tubulin. For this cells were washed twice with PBS and fixed for 10 min in 4% paraformaldehyde in PBS at 37°C. Immunocytochemistry was performed by first permeabilising the cells with 0.1% Triton X-100 in PBS for 30 min (room temperature), followed by a 1hour block using 5% bovine serum albumin in PBS. The cells were incubated with anti-βIII tubulin antibodies (1:1000, Promega) overnight at 4°C, washed three times in PBS and incubated with FITC-labelled rabbit anti-mouse IgG secondary antibody (Molecular Probes Inc, Eugene, OR, USA) for 2h30 at room temperature.

The neuritic arbours of 60 to 90 neurons were imaged and neurite morphology assessed using three different analyses. Total neurite length was assessed by counting the number of coloured pixels per neuron, using skeletonised neurons, and adjusted

by the number of pixels in a bar of known length, while the total number of branch points were counted manually. In addition to these basic analyses, the Sholl analysis was also used to gain a more detailed quantification of neuronal morphology (Sholl, 1953). For this analysis, computer-generated concentric rings 30 μm apart were superimposed on the image, centred on the cell soma (figure 2.3A). The number of neurites intersecting each ring was then counted. An example of the additional information that can be gained by such an analysis can be seen in figure 2.3B. The two theoretical arbors are of the same total neurite length and have the same number of branch points, but have very different morphologies. These differences are visible in the Sholl analysis. In each experimental condition all imaging and quantification was performed blind. The data shown for each analysis is from a single experiment that is representative of the results obtained in three separate experiments. Statistical analysis was performed using ANOVA followed by Fishers' post-hoc test.

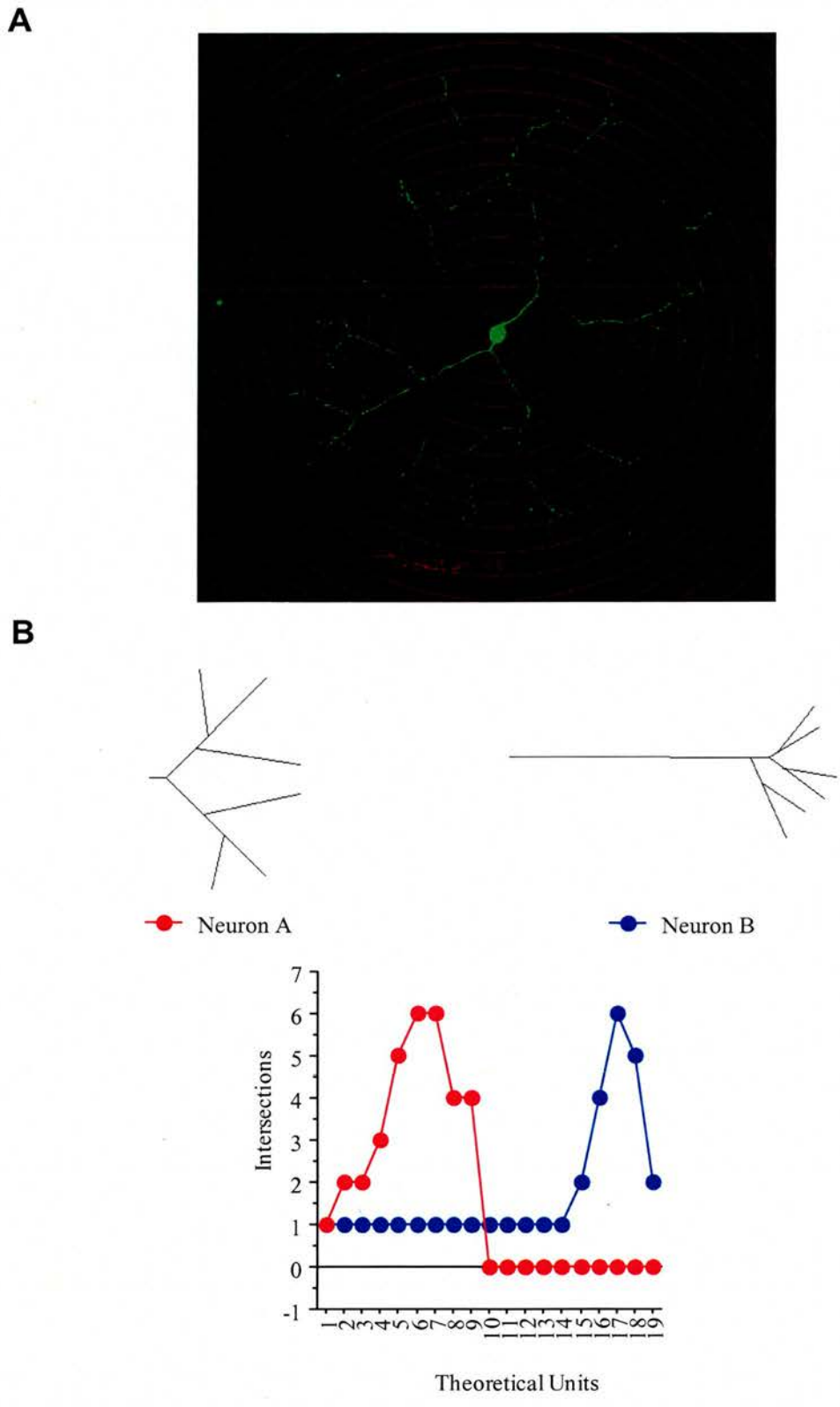


Figure 2.3 *Sholl analysis*
A) Photomicrograph of a pYFP transfected neonatal nodose neurons cultured for 24h in the presence of BDNF and overlaid with the concentric circles used to perform the Sholl analysis. B) Two theoretical neurons, of the same length and with the same number of branch points, but with two different Sholl analysis profiles.

2.3. Results

2.3.1. Phosphorylation and degradation of IκB-α is essential for neurite outgrowth and NFκB-activated gene transcription during a restricted window of development

2.3.1.1. Preventing NFκB-induced gene transcription by transfection of super-repressor IκB-α reduces the neurite growth of nodose neurons.

The investigation into the role of NFκB in the regulation of neuronal morphology began with a study of the effect of transfecting neonatal nodose neurons with a plasmid expressing a super-repressor form of the IκB-α protein. This plasmid produces an IκB-α protein capable of binding NFκB dimers, but which carries serine to alanine mutations at positions 32 and 36 that prevent the signal-induced phosphorylation required to target it for ubiquitination and proteosomal degradation. Production of this protein therefore blocks the release the NFκB dimers for nuclear translocation and consequently prevents NFκB induced gene transcription (Rodriguez et al., 1996; Roff et al., 1996).

NFκB is known to be involved in the survival of many types of neurons, therefore the first experiment assessed whether the transfection of neonatal nodose neurons with super-repressor IκB-α altered neuronal survival. Dissociated cultures of nodose neurons were established and, after three hours incubation in the absence of trophic support, transfected with either the super-repressor IκB-α or a control plasmid, in combination with a plasmid expressing YFP to allow visualisation of transfected neurons. Half the culture dishes were then supplemented with BDNF. Neurons were transfected by ballistic transfection, using plasmid-coated inert gold microcarriers. Twelve hours later, once all transfected neurons were clearly identifiable by their expression of YFP, an initial count of the number of transfected neurons was performed. A second count of living, YFP-positive neurons, performed 48 hours after transfection, was used to assess survival. The number of surviving YFP-positive neurons at 48 hours was expressed as a percentage of the initial number of

transfected neurons in each dish. Figure 2.4A shows that after 48 hours in culture in the presence of BDNF 80% of control-transfected neurons survived, and that transfection with the IκB-α super-repressor plasmid had no apparent effect on neuronal survival. At the same time, only 40% of neurons cultured in the absence of BDNF were surviving, and transfection with the IκB-α super-repressor had no additional effect on their survival (data not shown). These results suggest that in the case of neonatal nodose neurons surviving with BDNF there is no effect on survival as a consequence of blocking NFκB signalling through transfection of super-repressor IκB-α.

As super-repressor IκB-α transfection has no effect on the survival of nodose neurons, its role in neurite outgrowth can be easily assessed, as any effect seen cannot be attributed to differences in the survival of any subpopulations of neurons present. Cultures were established in the same manner as the survival cultures described earlier. Twenty-four hours after transfection 40-70 YFP-positive neurons per condition were digitally imaged using confocal microscopy and their neurite arbours reconstructed. Examples of reconstructed neurons grown in the presence of BDNF can be seen in figure 2.5. The images in the left column show the range of neurite arbours of control-transfected neurons, while the images in the right-hand column show the range of those transfected with super-repressor IκB-α. The neurons

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Figure 2.4 *Super-repressor IκB-α reduces neurite growth from neonatal nodose neurons but does not affect survival*
P1 nodose neurons were transfected with a YFP expression plasmid together with either a super-repressor IκB-α plasmid or an empty control plasmid and incubated in medium containing 10ng/ml BDNF. Control transfected neurons were also grown without BDNF. A) Percent survival 48 hours after transfection. B) Sholl analysis of neurite arbour morphology 24 hours after transfection. C) Total neurite length 24 hours after transfection. D) Number of branch points in neurite arbours 24 hours after transfection. E) Photomicrographs of a typical control and super-repressor IκB-α transfected neurons grown for 24 hours with BDNF (Scale bar = 50 μm). The means and standard errors of data obtained from 40-70 neurons in each experimental condition are shown. Statistical comparisons shown are with respect to the control transfected neurons, * $p<0.05$, ** $p<0.001$.

Figure 2.5 *Representative sample of reconstructed newborn nodose neurons*
The illustrated neurons represent the range of morphologies observed in P0 control transfected cultures (left) and super-repressor IκB-α transfected cultures (right) after 24 hours incubation with 10 ng/ml BDNF. The neurons shown correspond to percentiles 25, 50, 75 and 100 of the sampled populations in terms of total neurite length. Scale bar = 50 μm.

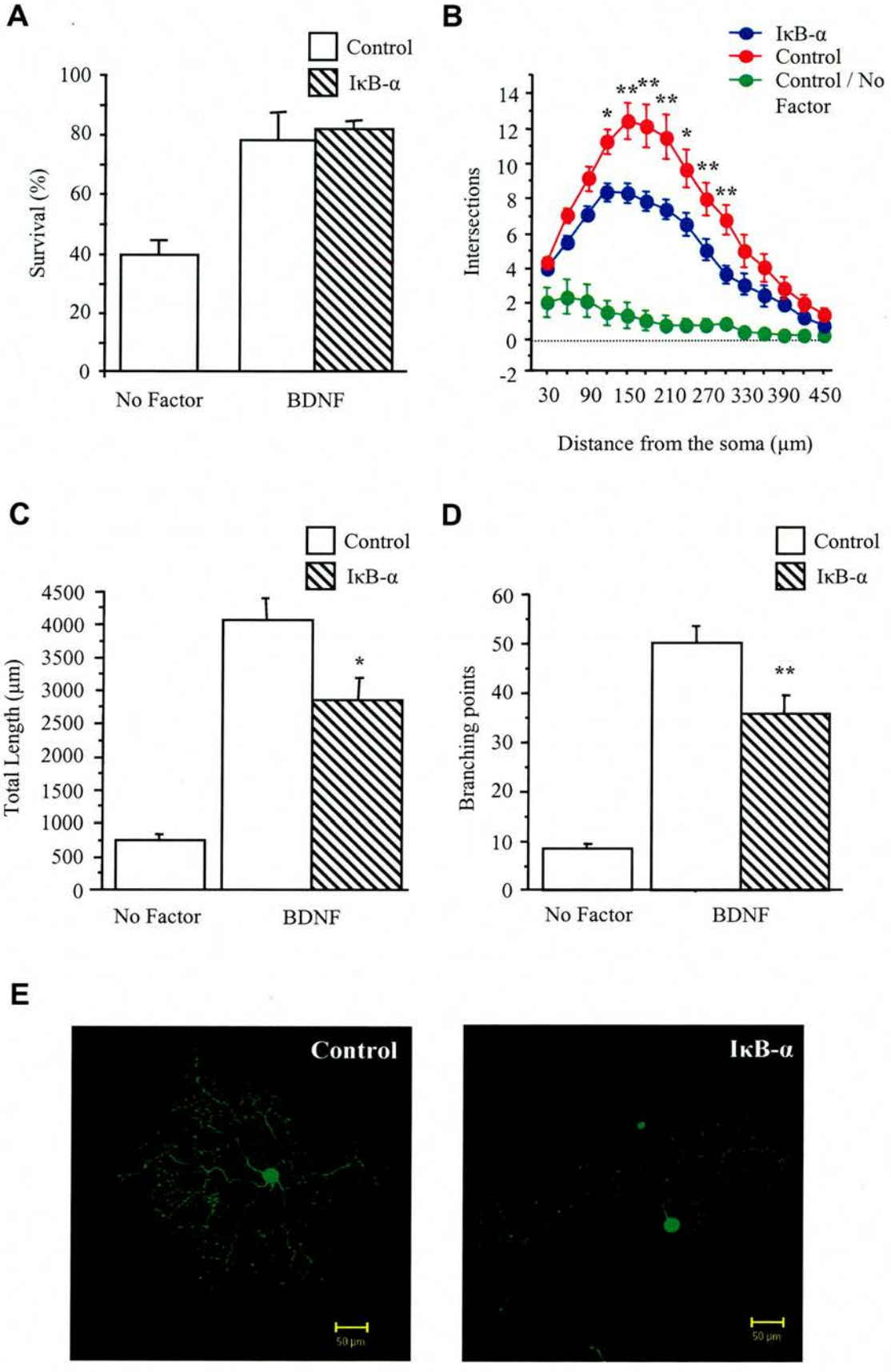
A
Control
IκB-α
Survival (%)
100
80
60
40
20
0
No Factor
BDNF
B
IκB-α
Control
Control / No Factor
Intersections
14
12
10
8
6
4
2
0
-2
30 90 150 210 270 330 390 450
Distance from the soma (μm)
C
Control
IκB-α
Total Length (μm)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
No Factor
BDNF
D
Control
IκB-α
Branching points
60
50
40
30
20
10
0
No Factor
BDNF
E
Control
IκB-α
50 μm
50 μm

Figure 2.4

Control **IκB-α**

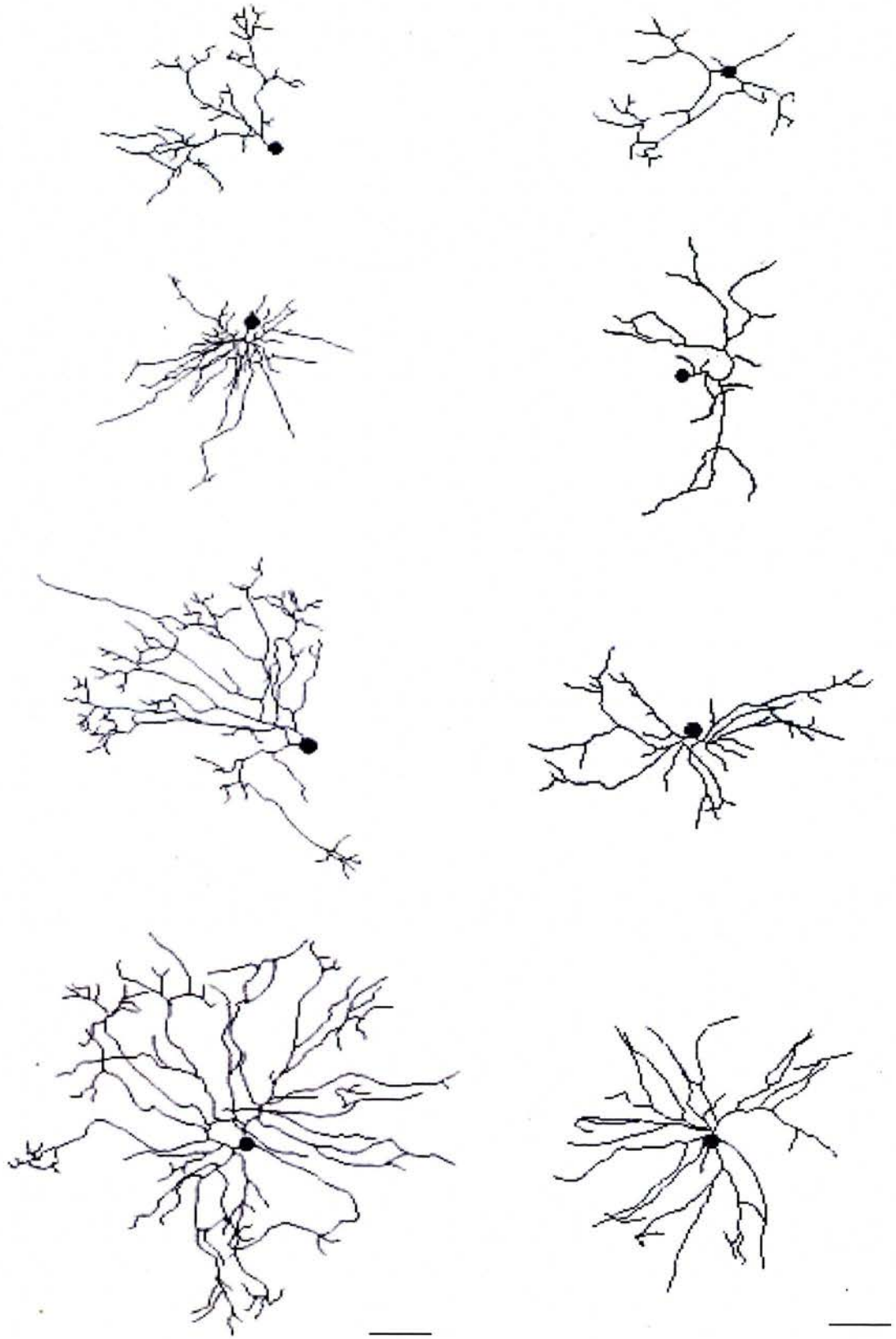

Figure 2.5

shown represent percentiles 25, 50 75 and 100 of the sampled populations in terms of total neurite length, and it can be seen that the neurite arbours of neurons transfected with super-repressor IκB-α appear smaller and less intricate than those transfected with the control plasmid.

Sholl analysis and assessment of total neurite length and number of branch points were performed on the reconstructed neurons. Figure 2.4B shows the Sholl analysis of P1 nodose neurons cultured for 24 hours in the presence of BDNF and transfected with either super-repressor IκB-α or a control plasmid. The number of neurites intersecting each ring of a series of digitally generated concentric circles, 30 μm apart and centred on the cell body, was counted, providing a quantification of the complexity of the neurite arbour at a range of distances from the cell body. Control transfected neurons cultured in the presence of BDNF show that, on average, the total number of intersections at each distance increases from a mean of 4.2 intersections at the first ring, 30μm from the cell body, to a maximum number of 12.4 intersections at 150μm, after which the number of intersection decreases so that by 450μm from the cell body less that 2 intersections can be found. In neurons transfected with the super-repressor IκB-α the number of intersections at each ring is decreased as compared to control neurons, with this effect reaching statistical significance at distances of between 120 and 300μm from the cell soma. Figure 2.4B also shows the outgrowth seen in control-transfected neurons cultured in the absence of trophic support, where a mean of 2 intersections per neurons can be seen at the initial ring 30μm from the cell body, and in which there is no subsequent peak, only a gradual decrease in the number of intersections detected per ring.

The effect of IκB-α on neurite outgrowth can also be seen when the total neurite length and number of branch points are calculated. These calculations were performed on the same set of neurons and the results can be seen in figures 2.4C and 2.4D (total neurite length and number of branch points, respectively). The total neurite length of P1 control neurons grown in the presence of BDNF was found to average 4100 μm, while the average length of neurons cultured in the absence of trophic support was found to be 750 μm. Neurons cultured in the presence of BDNF,

but transfected with super-repressor IκB-α, showed a 30.1% decrease in total neurite length as compared to those transfected with the control plasmid ($p < 0.05$, Student's t-test). Similarly control neurons cultured in the presence of trophic support possessed an average of 50 branch points per neuron, while those grown in the absence of trophic support possessed an average of only 8. Neurons cultured in the presence of BDNF, but transfected with the super-repressor IκB-α construct showed a 28.4% reduction in the average number of branch points compared to the control transfected neurons ($p < 0.001$, Student's t-test). Figure 2.4E shows examples of typical control and super-repressor IκB-α neurons grown in the presence of BDNF. To exclude the possibility that transfection with IκBα reduced overall cellular metabolism, and consequently decreased neurite outgrowth, the size of the cell soma was measured in super-repressor IκBα- and control-transfected cells grown in the presence of BDNF for 24h. The somal cross sectional area was quantified using confocal microscopy, revealing that there was no significant difference between the somal size of the super-repressor IκBα-transfected neurons ($680\pm113\mu m^2$, n=50) and control-transfected neurons ($715\pm34\mu m^2$, n=51). Taken together these results show that blocking NFκB-induced gene transcription by transfection with super-repressor IκB-α decreases the overall size and complexity of the neurite arbours of neonatal nodose neurons without affecting cell survival or causing a global reduction in cell metabolism affecting cell growth.

2.3.1.2. Basal NFκB dependent gene expression is not affected by BDNF

The next group of experiments addressed the question of whether BDNF is able to influence basal NFκB expression. To do this the levels of NFκB-dependent transcriptional activity in neonatal nodose neurons were measured under different experimental conditions by transfecting these neurons with a plasmid expressing GFP under the control of an NFκB promoter sequence. Three hours after plating, the neurons were transfected with this plasmid in combination with a plasmid expressing RFP (to allow identification of transfected neurons even in the absence of NFκB-dependent GFP transcription) and either the superrepressor IκB-α plasmid or the corresponding empty control vector. These neurons were then incubated in the

presence or absence of BDNF for 18 hours. Representative fields of neurons cultured in the presence of BDNF, and transfected with either the IκB-α super-repressor or control plasmid can be seen in Figure 2.6A. The upper panels show neurons identified through the RFP signal, superimposed with the NFκB-dependent GFP image, while the lower panels show the GFP signal alone. Neurons transfected with the control plasmid exhibit a clear GFP signal (left panels) while in the neurons transfected with super-repressor IκB-α only a weak expression of GFP can be seen. Note also that the decrease in neurite length and complexity found in the previous experiments can also been seen in the RFP image in this study. Quantification of the levels of fluorescence in these neurons revealed an 80% decrease in the levels of GFP fluorescence in neurons cultured in the presence of BDNF and transfected with super-repressor IκB-α as compared to control transfected neurons (Figure 2.6B). Also of interest is that there was no significant difference in the level of fluorescence in control-transfected neurons grown with or without BDNF and that super-repressor IκB-α-transfected neurons grown in the absence of trophic support showed a similar decrease in GFP fluorescence as those grown in the presence of trophic support.

Figure 2.6 *NFκB-dependent gene expression in newborn nodose neurons is not affected by BDNF.* P0 nodose neurons were co-transfected with the GFP NF-κB reporter plasmid, RFP plasmid and either the super-repressor IκB-α plasmid or corresponding control plasmid. They were then cultured with or without 20ng/ml BDNF for 24h. A) Photomicrographs of representative fields of neurons cultured with BDNF for 24h. The left panels show neurons transfected with IκB-α and the right panels show those transfected with a control plasmid. Transfected neurons, with their dendritic arbours are outlined by RFP in the upper panels. The effect of super-repressor IκB-α on neuronal morphology, seen in the previous figures, is also evident in these images. NFκB-dependent GFP fluorescence in the same fields (lower panels) shows the marked reduction caused by super-repressor IκB-α (Scale bar = 50 μm). B) Quantification of the level of NF-κB-driven GFP fluorescence in super-repressor IκB-α-transfected and control-transfected neurons after 24 hours incubation with and without 20 ng/ml BDNF. Fluorescence measurements were made from 40 to 60 neurons in each experimental condition, and the data are expressed as a percentage of the mean fluorescence of the No factor/Control vector-transfected group (mean and standard error are shown). Statistical comparisons shown are with respect to the control transfected neurons, ** $p<0.001$. C) Time course of NF-κB-driven GFP fluorescence after BDNF stimulation. Neurons were transfected with the GFP NFκB reporter plasmid and pRFP and cultured overnight in the absence of trophic support. Neurons were then imaged immediately prior to and at 0.5, 1, 2 and 3 hours after the addition of BDNF to the medium (20ng/ml). An untreated group of neurons (No Factor) was imaged at the same time. The fluorescence of each neuron was quantified and expressed as a percentage of the initial (0h time point) measurement for each group.

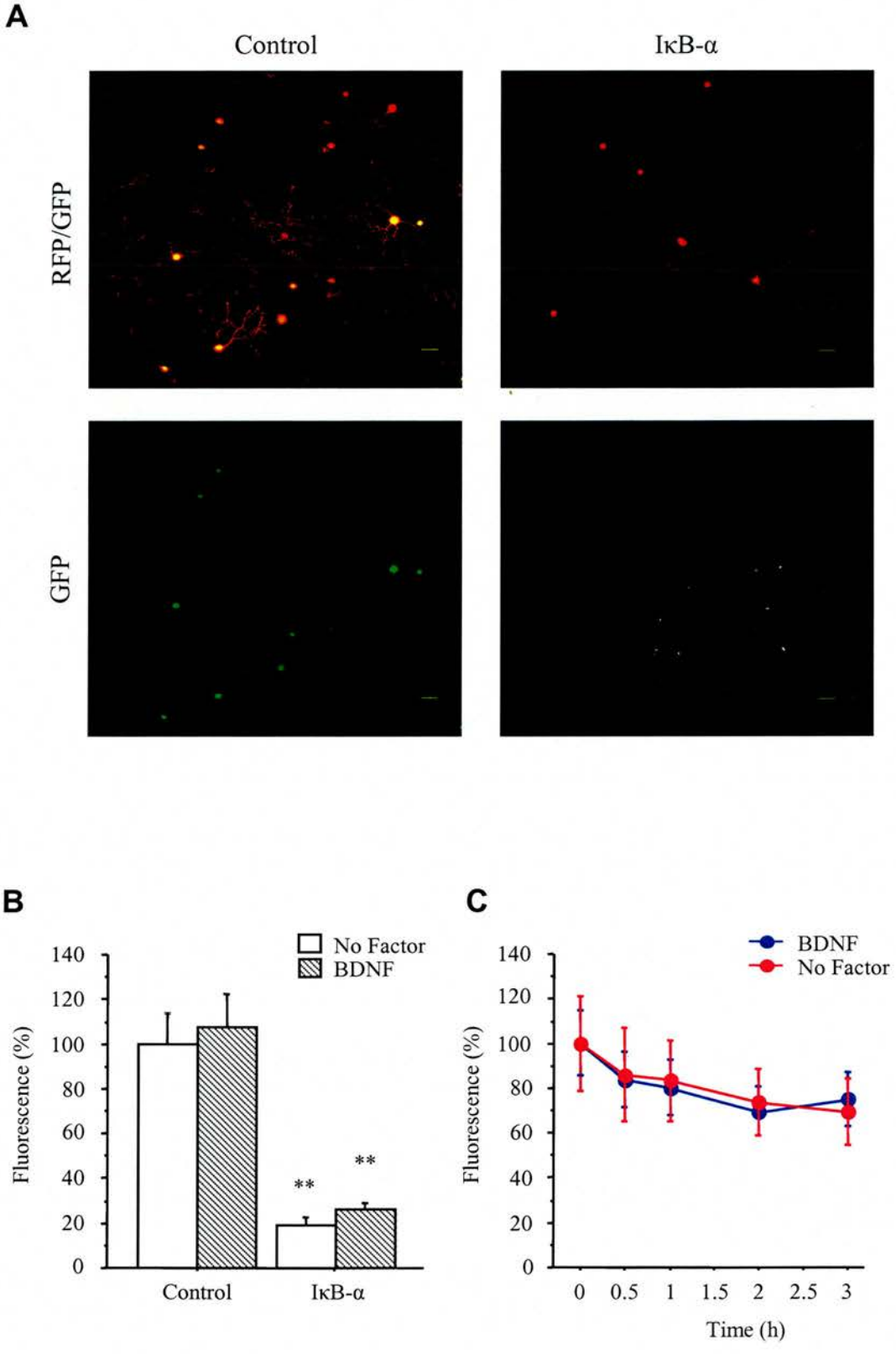
A
Control
IκB-α
RFP/GFP
GFP
B
No Factor
BDNF
Fluorescence (%)
140
120
100
80
60
40
20
0
**
**
Control
IκB-α
C
BDNF
No Factor
Fluorescence (%)
140
120
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3
Time (h)

Figure 2.6

To investigate further whether BDNF treatment is capable of activating NFκB-dependent gene transcription in this experimental situation, neurons were plated in defined medium, transfected with both pRFP and the plasmid expressing GFP under the control of the NFκB promoter and cultured overnight in the absence of trophic support. Identifiable fields in these cultures were then imaged both before and at 0.5, 1, 2 and 3 hours after treatment with BDNF, as were similar fields from untreated parallel cultures. Figure 2.6C shows that at no time point did BDNF treatment appear to increase NFκB-induced GFP expression above those of untreated neurons. These results therefore suggest that BDNF does not affect NFκB-induced gene transcription in this experimental paradigm.

2.3.1.3. The super-repressor IκB-α decreases the neurite outgrowth of nodose neurons in a developmentally dependent manner.

The response of nodose neurons to trophic factors is known to change significantly throughout development, particularly during the embryonic and early postnatal stages, during which neurons are establishing their patterns of innervation and neurotransmitter phenotypes (see Chapter 1). The next step was therefore to investigate whether the role of NFκB in promoting neurite outgrowth is similar throughout development. To this end, cultures of nodose neurons were established from the earliest age at which these neurons can be successfully transfected using the gene-gun (E16), up to P3. Neurons were then transfected with either a control plasmid or super-repressor IκB-α, along with pYFP, and half the dishes were supplemented with BDNF, as in the previous set of experiments. Again, neurons were imaged at 24 hours using a confocal microscope and their survival assessed by counting at 48 hours. Throughout the range of ages studied the survival of control-transfected neurons grown in the presence of trophic support was between 60 to 80%. In this case figure 2.7A shows the proportion of transfected neurons surviving to 48 hours for each dish expressed as a percentage of the mean value for the positive control group to allow comparison between different ages, each with differing inherent potential to survive in the presence of BDNF. It can be seen that there was no significant difference between the survival of neurons transfected with either the

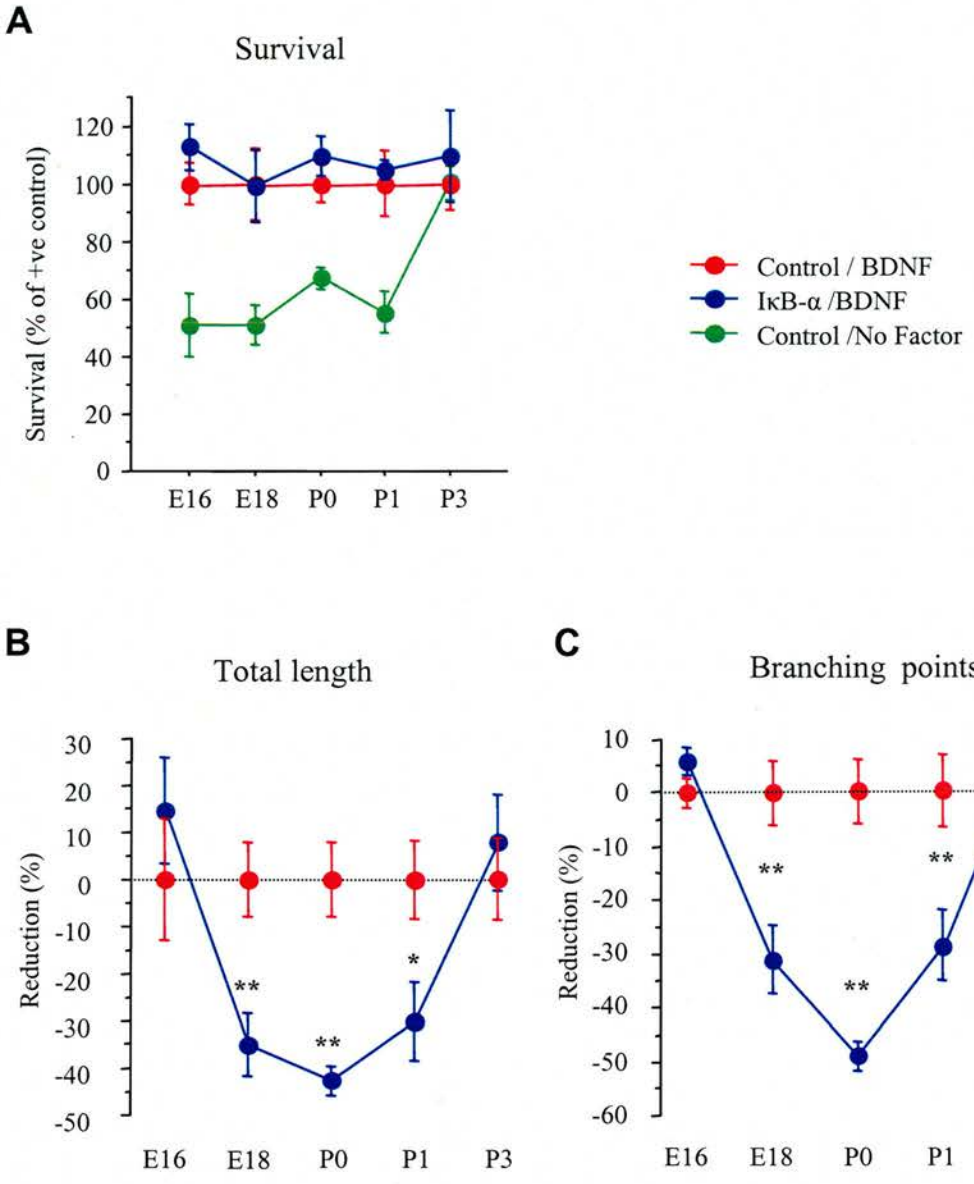

Figure 2.7 *Super-repressor IκB-α reduces neurite growth from nodose neurons in a developmentally dependent manner*

E16, E18, P0, P1 and P3 nodose neurons were transfected with a YFP expression plasmid together with either a super-repressor IκB-α plasmid or an empty control plasmid and were incubated in medium containing 10 ng/ml BDNF. A) Neuronal survival 48 hours after transfection, expressed as a percentage of the BDNF, control-transfected group at each age. In addition to showing survival data for super-repressor IκB-α transfected and control transfected neurons grown with BDNF, survival data for control-transfected neurons grown without BDNF are also shown at each age. B) Total neurite length 24 hours after transfection. C) Number of branch points in neurite arbors 24 hours after transfection. D-H) Sholl analysis of neurite arbour morphology 24 hours after transfection at E16, E18, P0, P1 and P3, respectively. The means and standard errors of data obtained from 60 neurons in each experimental condition are shown. Statistical comparisons shown are with respect to the control transfected neurons, * $p<0.05$, ** $p<0.001$.

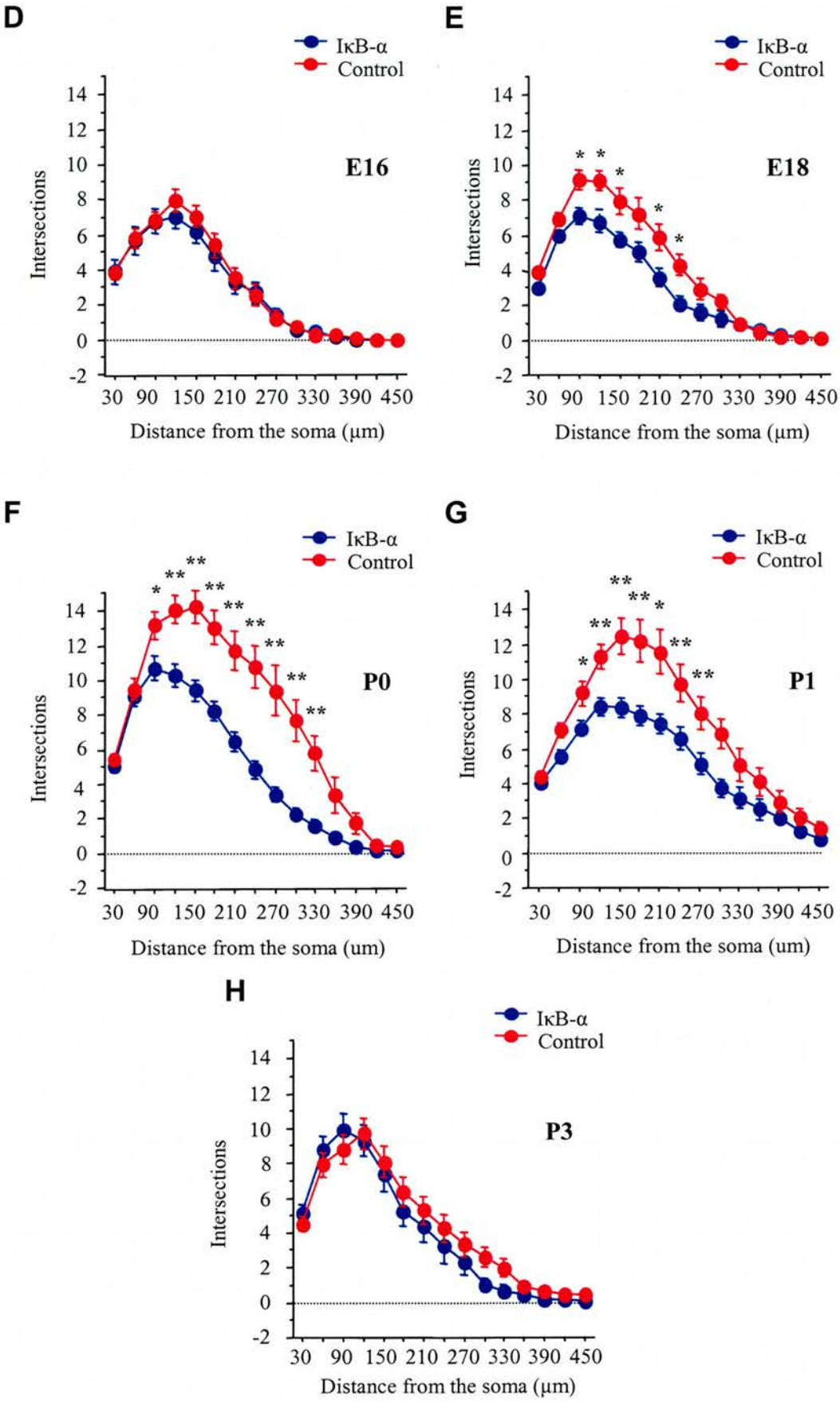
D
IκB-α
Control
E16
Intersections
Distance from the soma (μm)
E
IκB-α
Control
E18
Intersections
Distance from the soma (μm)
F
IκB-α
Control
P0
Intersections
Distance from the soma (um)
G
IκB-α
Control
P1
Intersections
Distance from the soma (um)
H
IκB-α
Control
P3
Intersections
Distance from the soma (μm)

Figure 2.7

control plasmid or superrepressor IκB-α, when grown in the presence of BDNF. Interestingly, anl increase in the survival of control transfected neurons cultured in the absence of BDNF can be seen until P3, when the neurons of the nodose ganglia appear to be capable of surviving in the absence of additional trophic support. Analysis of the total neurite length and branching showed that transfection of neurons at E16 and P3 with super-repressor IκB-α did not have any effect on the outgrowth of these neurons (Fig 2.7B and 2.7C respectively). However, during the perinatal period, between E18 and P1, an important effect of suppressing NFκB-induced gene transcription was revealed. This effect peaked in cultures established from pups on the day of birth, where a 43% decrease in neurite length and 48% decrease in neurite branching was seen ($p < 0.001$, Student's t-test). These results were also reflected in the Sholl analyses (Fig 2.7 D-H) where significant decreases in the complexity of the neurite arbour were seen in neurons cultured at E18, P0 and P1. Together, these results suggest that NFκB-induced gene transcription plays a role in neurite outgrowth in a developmentally restricted manner, having an effect only during late embryonic and early postnatal stages.

2.3.1.4. BAY 11-7082 reduces neurite outgrowth from nodose neurons

To confirm the role of NFκB in the growth of neuritic arbours a variety of methods were employed, all of which were targeted at disrupting NFκB signalling. The second method used was disrupting IκB-α phosphorylation with BAY 11-7082, a commonly used, selective and irreversible inhibitor of IκB-α phosphorylation (Chiarugi, 2002; Pierce et al., 1997). These experiments were performed using P0 nodose neurons, as the earlier study indicated that this was the age at which the effect of blocking NFκB-activated gene transcription was maximal. The use of a chemical inhibitor also allowed us to examine the role of NFκB in neurite outgrowth in low density cultures, where there is no contact between neurons, as opposed to the earlier transfected cultures where close contact between YFP-positive neurons and adjacent untransfected neurons may alter neuronal morphology. To investigate whether this dose of BAY 11-7082 affected neurite outgrowth or neuronal survival, neurons were plated and after three hours subjected to a 45 minute pre-incubation with BAY 11-

7082 or a similar volume of vehicle, before the addition of BDNF to half of the dishes. Figure 2.8A shows that there was no significant difference in the survival of neurons grown in BDNF-supported cultures and treated with either vehicle or 10μM BAY 11-7082, at 24 hours after plating. Immediately after the second survival count, at 24h, neurons were fixed and stained for βIII-tubulin expression, combined with a fluorescent secondary antibody, to allow imaging using confocal microscopy. These images were analysed and the results can be seen in Figures 2.8B-E. The Sholl analysis (figure 2.8B) shows that treatment of P0 nodose neurons with BAY 11-7082 causes a decrease in the number of intersections at each ring, which reaches significance between 180 and 420μm from the cell body. This finding is reflected in the analyses of total neurite length and number of branch points where treatment with BAY 11-7082 resulted in a 19.6% reduction in total neurite length and a 28.2% decrease in the number of branch points, both of which reached statistical significance ($p < 0.001$, Fig 2.8C and D respectively). It should also to be noted that neurons cultured at this low density appear to present a different pattern of outgrowth and branching as compared to the higher density cultures used for ballistic transfection. Whereas the high density cultures show a peak number of intersections between 90 and 150μm from the cell body at this age, neurons cultured at a lower density appears to have a less abrupt peak that occurs further from the cell body, here at 240μm, with the maximum number of intersections being lower, at around 7 intersections at the peak, whereas up to 14 intersections at the peak distance can be observed in the higher density cultures. This suggests that in low density culture neurites extend further before branching and that the distances between branches may also be longer, but that they also branch less than neurons in high density

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Figure 2.8. *BAY 11-7082 reduces neurite outgrowth from nodose neurons*
P0 nodose neurons were incubated in medium containing 10ng/ml BDNF and either the IκB-α phosphorylation inhibitor BAY 11-7082 at a concentration of 10μM or vehicle control. Vehicle treated neurons were also grown without BDNF. A) Percent survival after 48h in culture. B) Sholl analysis of neurite arbor morphology after 24h in culture. C) Total neurite length after 24h in culture. D) Number of branch points in neurite arbors after 24h in culture E) Photomicrographs of typical βIII tubulin stained, vehicle or BAY 11-7082 treated neurons grown for 24 hours with BDNF (Scale bar = 50 μm). The means and standard errors of data obtained from at least 40 neurons in each experimental condition shown. Statistical comparisons shown are with respect to the control transfected neurons cultured in the presence of BDNF, * $p<0.05$, ** $p<0.001$.

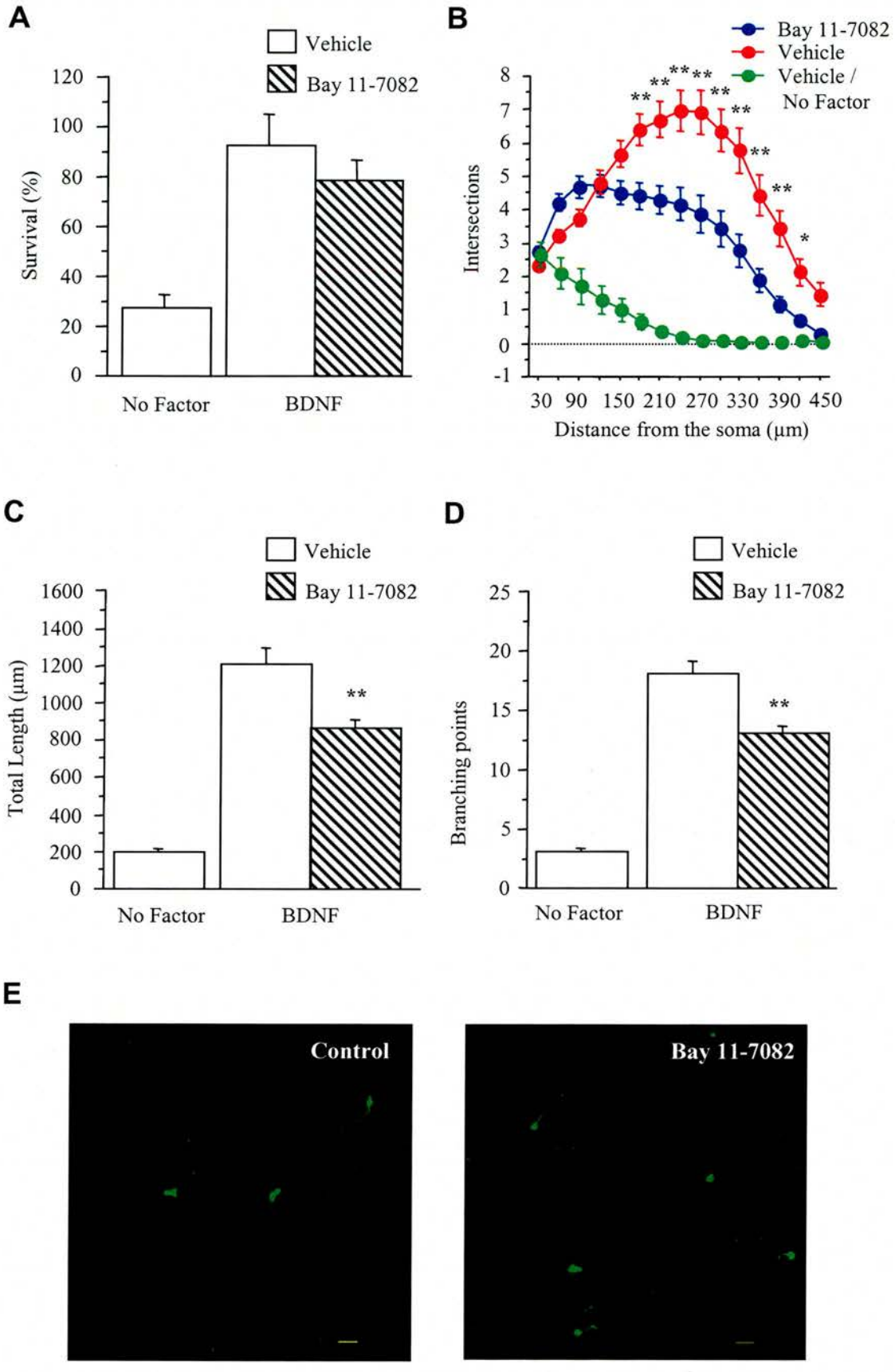
A
Vehicle
Bay 11-7082
Survival (%)
120
100
80
60
40
20
0
No Factor
BDNF
B
Bay 11-7082
Vehicle
Vehicle / No Factor
Intersections
8
7
6
5
4
3
2
1
0
-1
30 90 150 210 270 330 390 450
Distance from the soma (μm)
C
Vehicle
Bay 11-7082
Total Length (μm)
1600
1400
1200
1000
800
600
400
200
0
No Factor
BDNF
D
Vehicle
Bay 11-7082
Branching points
25
20
15
10
5
0
No Factor
BDNF
E
Control
Bay 11-7082

Figure 2.8

cultures. The images in figure 2.8E show typical neurons grown in the presence of trophic support, with and without BAY 11-7082. These results provide additional evidence supporting the conclusion that inhibition of NFκB signalling by interfering with the phosphorylation and degradation of IκB-α decreases neurite outgrowth from nodose neurons grown in both high and low density culture.

2.3.1.5. Proteosome inhibition reduces neurite outgrowth from nodose neurons

After phosphorylation by the IKK complex, IκB-α is ubiquitinated, targeting it for degradation by the proteosome. Therefore in order to provide additional support for the role of blocking NFκB signalling, at the level of the IκB, in causing a reduction in neurite outgrowth, the effects of the widely used proteosomal degradation inhibitor N-acetyl-leucinyl-leucinyl-norleucinal (ALLN) were studied (Chiarugi, 2002; Maggirwar et al., 1998). Cultures were established and analysed in the same fashion as those used to study the BAY 11-7082 compound and figure 2.9A shows that at a dose of 10μM ALLN has no effect on the survival of P0 nodose neurons. Analysis of individual neurons treated with the same dose of ALLN, however, revealed that there was a highly significant decrease in the number of intersections at all distances between 120 and 390μm from the cell body, with ALLN completely eliminating the increase in the number of intersections per ring that would normally occur up to approximately 210μm from the centre (figure 2.9B). This effect can also be seen in the analyses of total neurite length and branching (fig 2.9C and D), where treatment with ALLN reduces total neurite length by nearly 60% and branching by 65% ($p < 0.001$, Students t-test).

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* **Figure 2.9** *Proteosome inhibition reduces neurite growth from nodose neurons*
P0 nodose neurons were incubated in medium containing 10ng/ml BDNF and either the proteosomal degradation inhibitor ALLN at a concentration of 10μM or vehicle control. Vehicle treated neurons were also grown without BDNF. A) Percent survival after 48h in culture. B) Sholl analysis of neurite arbor morphology after 24h in culture. C) Total neurite length after 24h in culture. D) Number of branch points in neurite arbors after 24h in culture. E) Photomicrographs of typical βIII tubulin stained, vehicle or ALLN treated neurons grown for 24 hours with BDNF (Scale bar = 50 μm). Statistical comparisons shown are with respect to the control transfected neurons cultured in the presence of BDNF, * $p<0.05$, ** $p<0.001$.

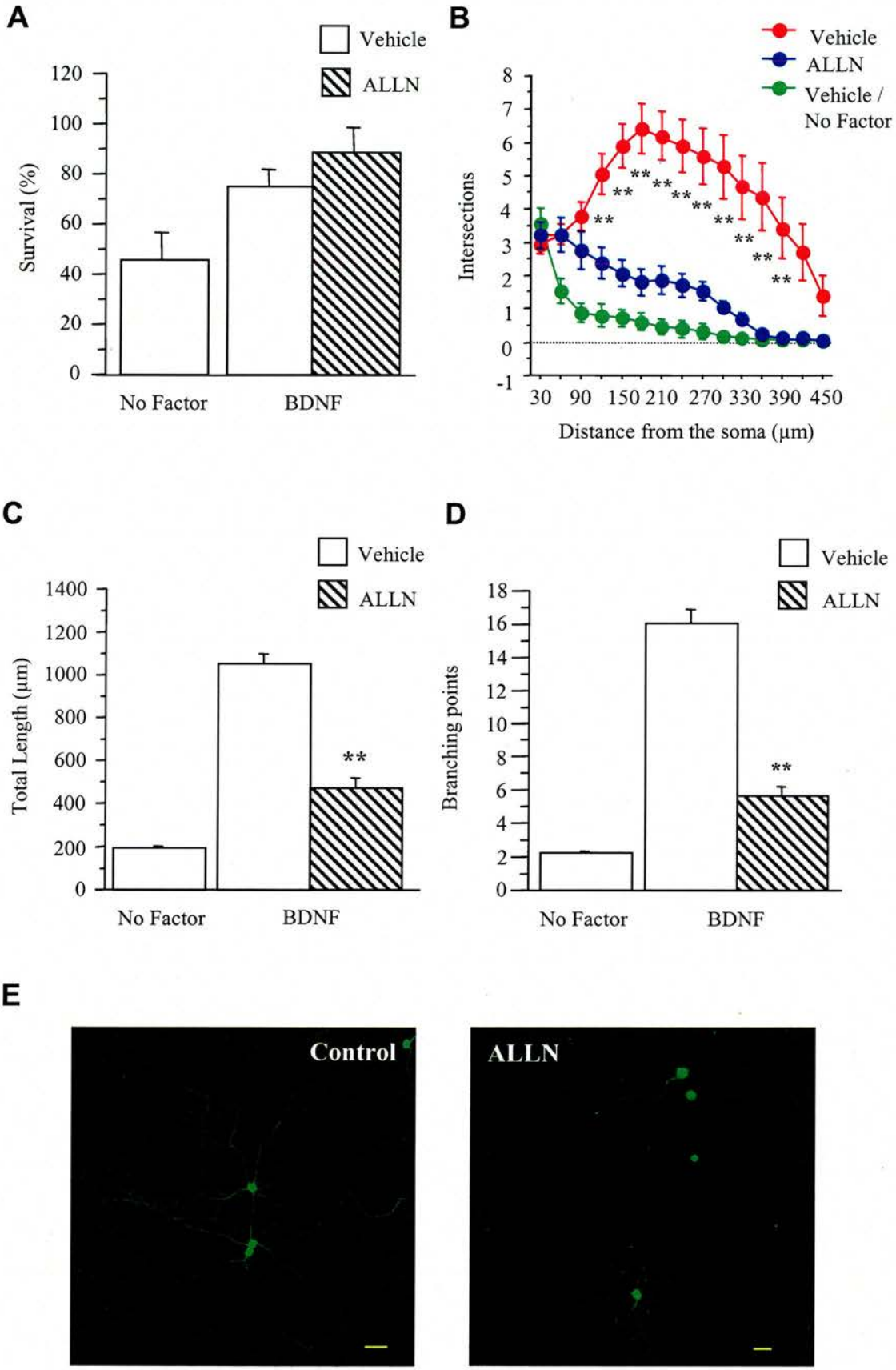
A
Vehicle
ALLN
Survival (%)
120
100
80
60
40
20
0
No Factor
BDNF
B
Vehicle
ALLN
Vehicle /
No Factor
Intersections
8
7
6
5
4
3
2
1
0
-1
**
30 90 150 210 270 330 390 450
Distance from the soma (µm)
C
Vehicle
ALLN
Total Length (µm)
1400
1200
1000
800
600
400
200
0
**
No Factor
BDNF
D
Vehicle
ALLN
Branching points
18
16
14
12
10
8
6
4
2
0
**
No Factor
BDNF
E
Control
ALLN

Figure 2.9

2.3.2. Disrupting NFκB-induced gene transcription using κB decoy DNA reduces neurite outgrowth in postnatal nodose neurons

As well as establishing that disrupting key steps in the activation of NFκB results in decreased neurite outgrowth from perinatal nodose neurons, the effect of directly blocking NFκB transcriptional activity in these neurons was also examined. This was done using κB decoy DNA. This is a double stranded oligonucleotide designed to contain a κB consensus binding sequence found in the promoters of NFκB target genes, and which has been previously described as preventing NFκB transcriptional activity both in vitro and in vivo (Yeh et al., 2002). This oligonucleotide, or a scrambled control oligo, were precipitated onto gold microcarriers, in combination with a YFP reporter to allow identification and visualisation of transfected neurons, and cultures of P0 neurons established and transfected in the same manner as that described for super-repressor IκB-α transfected cultures. Again, the survival of these cultures was assessed to rule out any detrimental effects on neuronal survival biasing the results. The transfection of κB decoy oligonucleotides was found to have no effect on the neuronal survival, as compared to the scrambled oligo control (Fig 2.10A), with the overall survival of both groups of neurons being excellent. Figure 2.10B shows the Sholl analysis of the neurons from these cultures, where the transfection of neurons with κB decoy DNA significantly reduced the number of neurites intersecting each ring from 120 to 360μm from the cell body ($p<0.05$ for 120μm, $p<0.001$ at all other distances) as compared to the scrambled oligo control. This difference is also reflected in a 27% decrease in total neurite length and a 36% decrease in the number of branch points. Typical neurons grown transfected with

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Figure 2.10 *κB decoy DNA reduces neurite growth from nodose neurons*
P0 nodose neurons were transfected with a YFP expression plasmid together with either a κB decoy DNA or a scrambled control variant and were incubated in medium containing 10 ng/ml BDNF. Control transfected neurons were also grown without BDNF. A) Percent survival 48 hours after transfection. B) Total neurite length 24 hours after transfection. C) Number of branch points in neurite arbours 24 hours after transfection. D) Sholl analysis of neurite arbour morphology 24 hours after transfection. E) Photomicrographs of typical neuron transfected with the scrambled control or κB decoy oligonucleotides and grown for 24 hours with BDNF. Scale bar = 50 μm. Statistical comparisons shown are with respect to the control transfected neurons cultured in the presence of BDNF, * $p<0.05$, ** $p<0.001$.

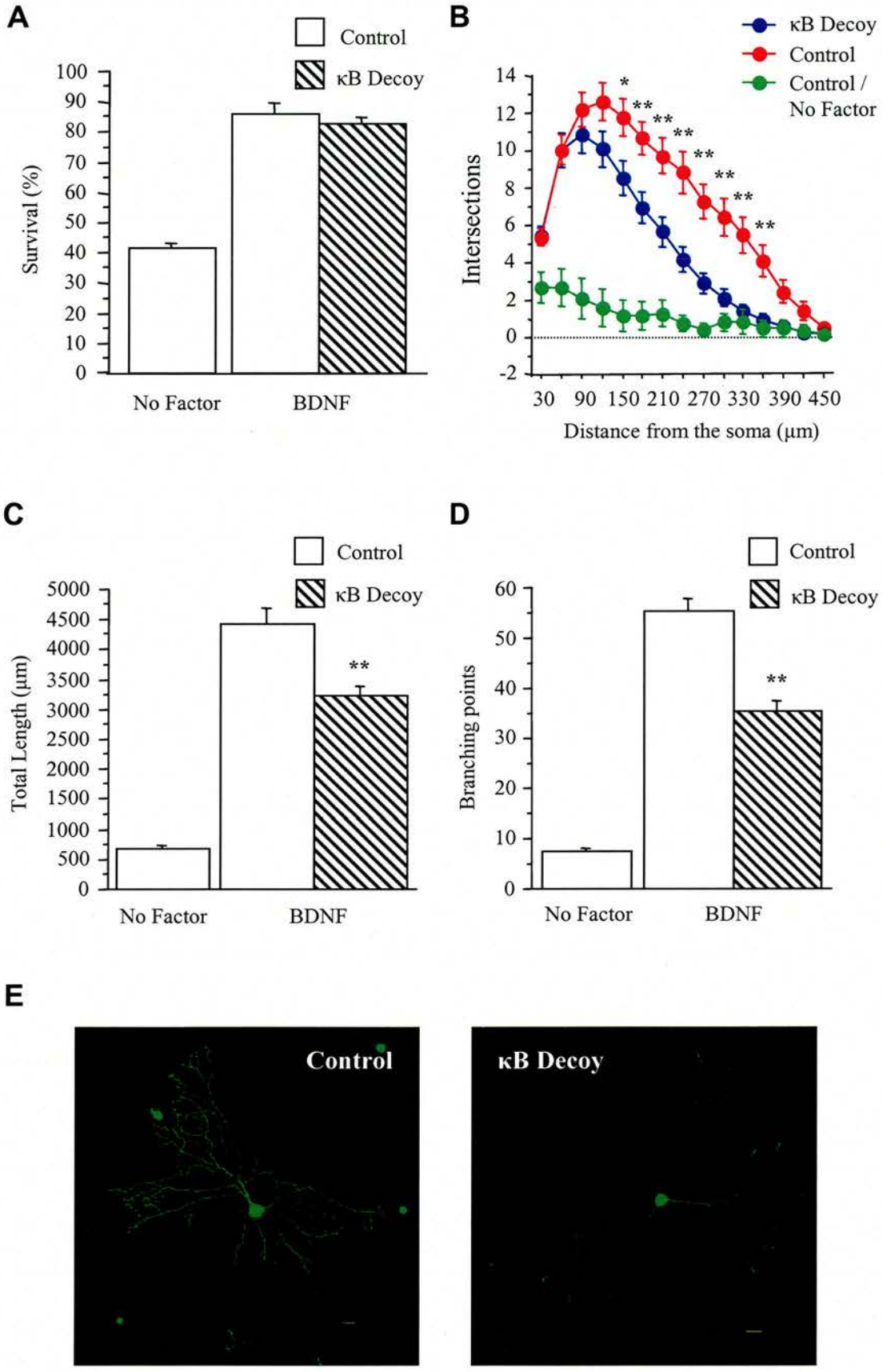
A
Control
κB Decoy
Survival (%)
100
90
80
70
60
50
40
30
20
10
0
No Factor
BDNF
B
κB Decoy
Control
Control /
No Factor
Intersections
14
12
10
8
6
4
2
0
-2
*
**
30 90 150 210 270 330 390 450
Distance from the soma (μm)
C
Control
κB Decoy
Total Length (μm)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
**
No Factor
BDNF
D
Control
κB Decoy
Branching points
60
50
40
30
20
10
0
**
No Factor
BDNF
E
Control
κB Decoy

Figure 2.10

either κB decoy DNA or the scrambled oligonucleotide and cultured in the presence of trophic support can be seen in Figure 2.10E. These results demonstrate that NFκB transcriptional activity also plays a role in controlling neurite outgrowth in P0 nodose neurons.

2.3.3. NFκB gain-of-function promotes neuronal survival, but has no effect on neurite outgrowth

To investigate whether increases in NFκB activity could promote additional neurite outgrowth in neonatal nodose neurons, P0 nodose neurons were established in culture and transfected with a p65 expression plasmid that has previously been shown to activate NFκB-induced gene transcription in other neurons (Hamanoue et al., 1999). Firstly we confirmed that expression of the p65 plasmid resulted in increased NFκB-induced gene transcription in our system. This was done by using the gene gun to simultaneously transfect neurons with the p65, or an empty control plasmid, in combination with the GFP/NFκB reporter plasmid used in section 2.3.1.2. This revealed that transfection of neonatal nodose neurons with the p65 expression plasmid caused a two-fold increase in NFκB-dependent gene transcription after 18 hours in culture (Fig 2.11A). Despite this, no changes in the growth and complexity of the neurite arbours were seen in p65 transfected neurons, either in the presence or absence of BDNF, in any of our analyses (Fig 2.11 C, D and E). Interestingly, one effect of increasing NFκB activity in these neurons was seen. While overexpression of p65 had no effect on neuronal survival when neurons were cultured in the presence of BDNF, in the absence of BDNF the two-fold increase in NFκB-dependent gene transcription was sufficient to cause neurons to survive to the

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* **Figure 2.11** *Overexpression of p65 increases NFκB activation and promotes neuronal survival but does not enhance neurite outgrowth*
A) Quantification of the level of NFκB-driven GFP fluorescence in neurons transfected with either a p65 overexpressing plasmid or the corresponding empty control, after being cultured in the presence of BDNF for 24 hours. B-E) P0 neurons transfected with either the p65-overexpressing or the empty control plasmid were cultured in the presence or absence of BDNF. B) Percent survival after 48h in culture. C) Sholl analysis of neurite arbour morphology after 24h in culture. D) Total neurite length after 24h in culture. E) Number of branch points in neurite arbours after 24h. Statistical comparisons are shown with respect to the control transfected neurons, ** $p<0.001$

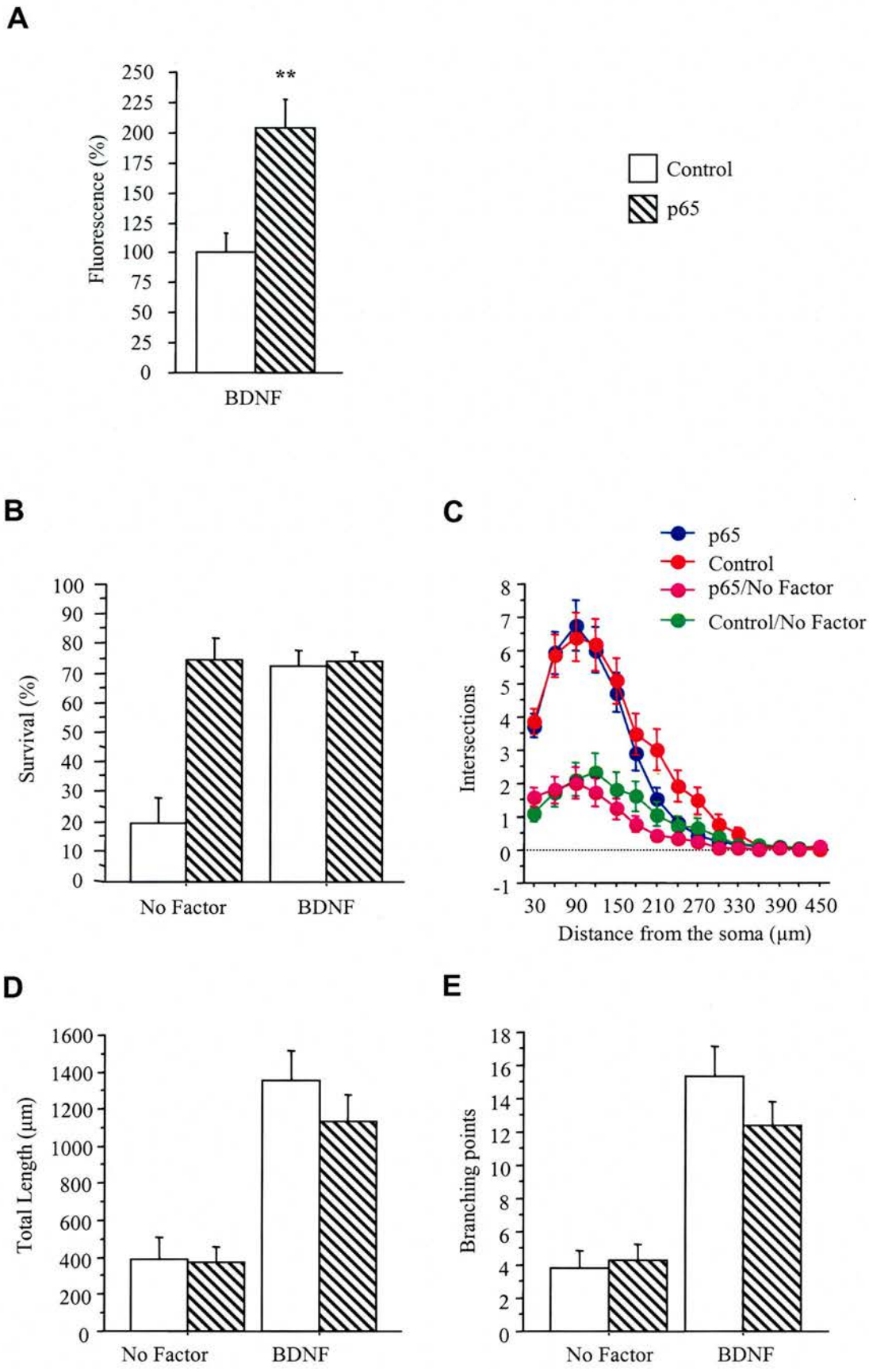
A
250
225
200
175
150
125
100
75
50
25
0
Fluorescence (%)
**
BDNF
Control
p65
B
100
90
80
70
60
50
40
30
20
10
0
Survival (%)
No Factor
BDNF
C
p65
Control
p65/No Factor
Control/No Factor
8
7
6
5
4
3
2
1
0
-1
Intersections
30 90 150 210 270 330 390 450
Distance from the soma (µm)
D
1600
1400
1200
1000
800
600
400
200
0
Total Length (µm)
No Factor
BDNF
E
18
16
14
12
10
8
6
4
2
0
Branching points
No Factor
BDNF

Figure 2.11

same extent as they do in the presence of BDNF (Fig 2.11B). These results suggest that the basal level of NFκB expression in nodose neurons is maximal for neurite growth, but suboptimal for survival. They also suggest that a BDNF-activated signalling pathway must cooperate with NFκB to promote neuronal survival in perinatal nodose neurons, as neurite extension is not seen in the neurons that survive in the absence of trophic support after transfection with p65.

2.3.4. Upstream of IκB-α, the outgrowth of early postnatal nodose neurons is independent of IKKα and NIK.

After demonstrating that NFκB activation involved in the growth and elaboration of neuronal processes in perinatal nodose neurons, two of the proteins that have been reported to be upstream of IκB-α phosphorylation and degradation were investigated. The first protein investigated was IKKα, a protein involved in the kinase complex that phosphorylates IκBα thus targeting it for degradation in the classical NFκB activation pathway. IKKα may also phosphorylate histone H3 to allow NFκB-dependent gene transcription (Anest et al., 2003; Birbach et al., 2002; Hayden and Ghosh, 2004; Yamamoto et al., 2003), as well as activating the non-canonical NFκB signalling pathway that releases p52-RelB dimers through the partial degradation of p100 (Solan et al., 2002; Xiao et al., 2001; Yilmaz et al., 2003). We used IKKα knockout mice to investigate the role of this protein in neurite outgrowth in nodose neurons. IKKα$^{-/-}$ embryos survive until just before birth. Neurite outgrowth from neurons derived from E18 embryos homozygous for a null mutation of the IKKα gene was quantified, and the results compared to wild-type littermates. Embryos were removed from the dam and following genotyping, the dissected homozygous mutant and wild-type ganglia grouped separately and plated in low density cultures. Survival was assessed from two counts of living neurons, at 3 and 24h, with half of the dishes of each genotype being supplemented with BDNF immediately after the three hours count, the remaining dishes being left without trophic support. At 24 hours the survival was assessed, immediately prior to fixing and staining for βIII-tubulin to allow images of the neurite arbours to be obtained using confocal microscopy. No significant difference was found between the survival of IKK-α knockout and wild-type neurons cultured with trophic support (figure 2.12A). There

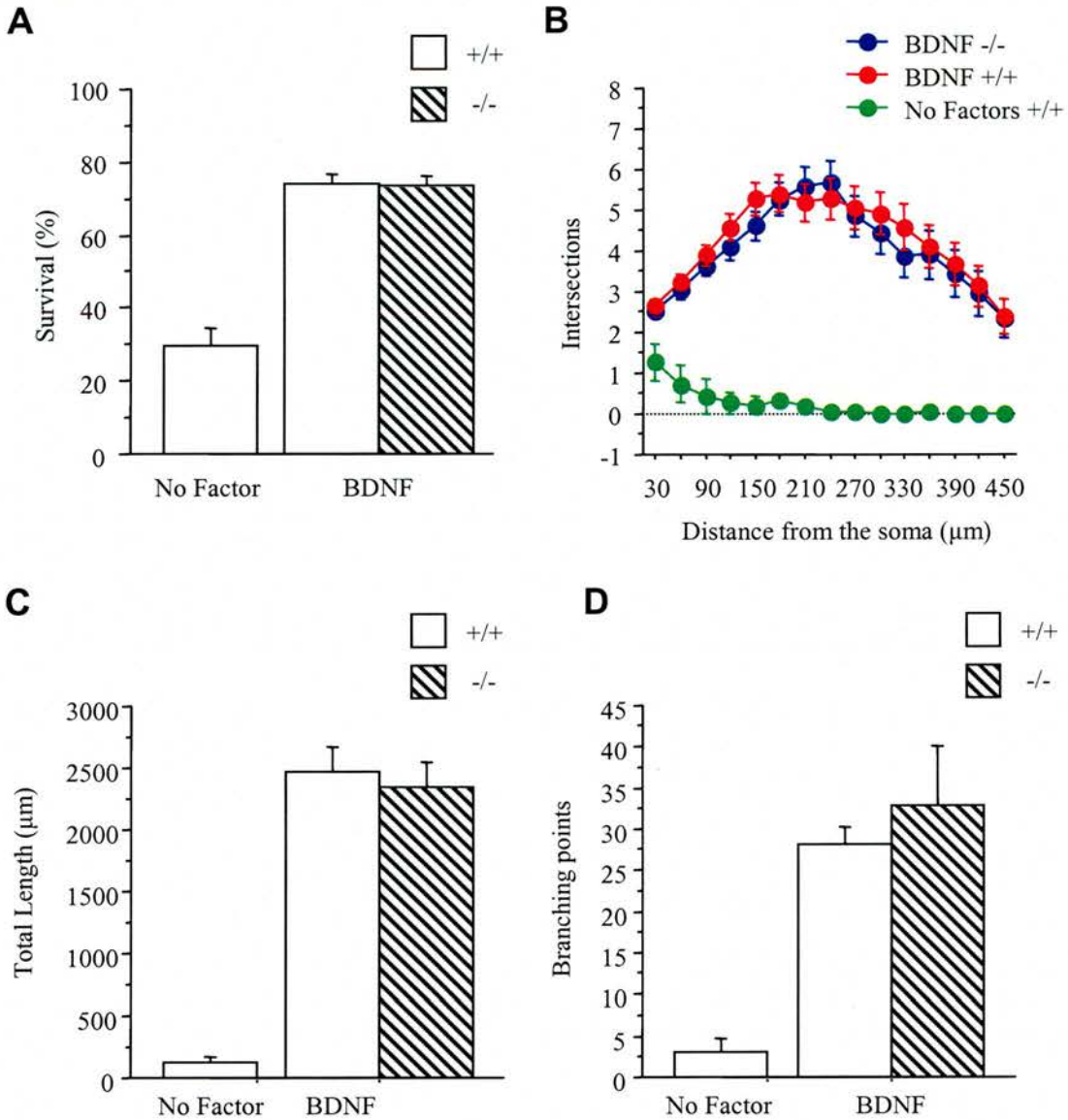

Figure 2.12 *Neurite outgrowth from IKK-α$^{-/-}$ nodose neurons is normal*
Nodose neurons from E18 IKK-α knockout mice were cultured for 24h in the presence of BDNF (10ng/ml) and their survival and outgrowth compared to wildtype littermates. A) Percent survival 48h after transfection. B) Sholl analysis of neurite arbour morphology 24h after transfection. C) Total neurite length 24h after transfection. D) Number of branch points in neurite arbors 24h after transfection. The means and standard errors of data obtained from 60-90 neurons in each experimental condition are shown.

was also no detected difference between the neurite outgrowth of IKK-α deficient and wild-type neurons in any of the analyses performed (Sholl analysis, total neurite length and number of branch points, figures 2.12B, C and D respectively). This suggests that IKK-α is not involved in activating NFκB in the regulation of neurite outgrowth. Unfortunately it was not possible to investigate whether the related protein IKKβ is involved in promoting neurite outgrowth in nodose neurons using the IKKβ knockout mice as these embryos die *in utero* at E14, an age at which no effect of NFκB on neurite outgrowth can be detected.

NFκB-inducing kinase (NIK) is another protein kinase that has been shown to play a role in activating NFκB signalling in cortical neurons (Bhakar et al., 2002) and may be involved in transducing the classical NFκB signalling pathway. NIK also activates IKKα in the non-canonical NFκB signalling pathway. The possibility of NIK being involved in activating the NFκB signalling responsible for neurite outgrowth in our culture system was investigated by transfecting P0 nodose neurons with either a construct expressing a dominant-negative form of NIK or a control plasmid in combination with pYFP in the same manner as described for super-repressor IκB-α earlier, and the survival and neurite outgrowth quantified. Transfection with dominant-negative NIK had no effect on neuronal survival in these cultures (figure 2.13A). No effect of dominant-negative NIK transfection on neurite outgrowth could be detected using either Sholl analysis or measurement of total neurite length or branching (figures 2.13B, C and D respectively). I also investigated whether the overexpression of wild-type NIK alters neurite outgrowth in these cultures. Using the same techniques, P0 nodose neurons were transfected with either wild-type NIK or a control plasmid and assessed for survival and neurite outgrowth. Figure 2.14A shows that transfection with wild-type NIK neither increased nor decreased neuronal survival, either in the presence or absence of BDNF. The outgrowth and complexity of NIK-overexpressing neurons grown in the presence of BDNF was identical to the outgrowth and complexity of control-transfected neurons, as can be seen in figures 2.14B, C and D (Sholl analysis, total neurite length and number of branch points respectively). These results suggest that NIK is not involved in activating NFκB in either of its roles in promoting the outgrowth or survival of nodose neurons.

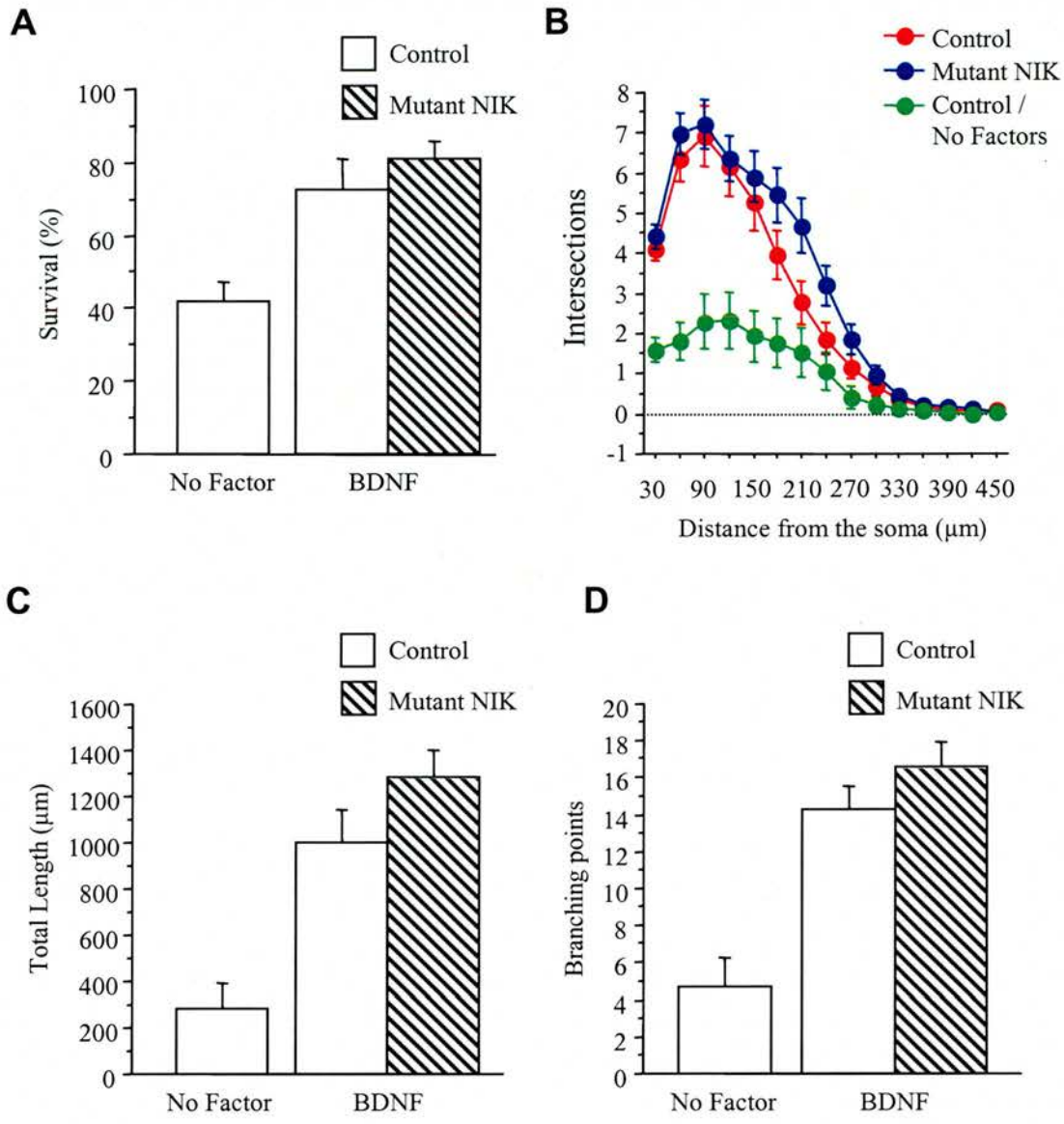

Figure 2.13 *Dominant-negative NIK does not affect neurite growth or survival in neonatal nodose neurons*

P0 nodose neurons were transfected with a YFP expression plasmid together with either a dominant-negative NIK plasmid or an empty control plasmid and incubated in medium containing 10 ng/ml BDNF. Control transfected neurons were also grown without BDNF. A) Percent survival 48 hours after transfection. B) Sholl analysis of neurite arbor morphology 24 hours after transfection. C) Total neurite length 24 hours after transfection. D) Number of branch points in neurite arbors 24 hours after transfection. The means and standard errors of data obtained from 60-90 neurons in each experimental condition are shown.

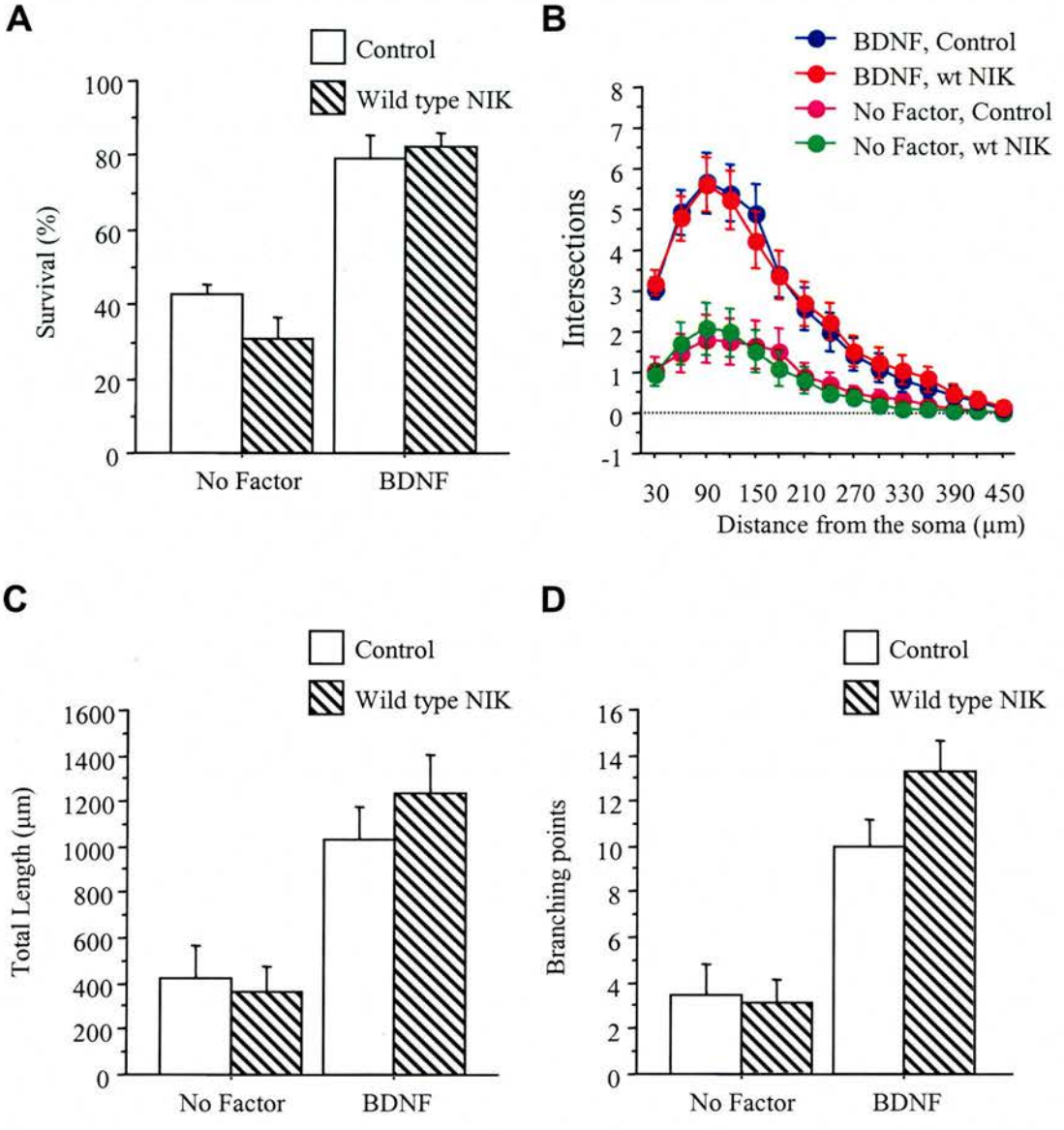

Figure 2.14 *Overexpression of wildtype NIK does not affect neurite growth or survival in neonatal nodose neurons*

P0 nodose neurons were transfected with a YFP expression plasmid together with either a wild-type NIK expressing plasmid or an empty control plasmid and were incubated in medium containing 10 ng/ml BDNF. Control transfected neurons were also grown without BDNF. A) Percent survival 48 hours after transfection. B) Sholl analysis of neurite arbor morphology 24 hours after transfection. C) Total neurite length 24 hours after transfection. D) Number of branch points in neurite arbors 24 hours after transfection. The means and standard errors of data obtained from 40-70 neurons in each experimental condition are shown.

2.4. Discussion

This study investigated whether the transcription factor NFκB plays a developmental role in regulating neurite outgrowth from peripheral sensory neurons of the nodose ganglia. Using several complementary methods it was found that inhibiting NFκB activation or NFκB-dependent transcriptional activity in neonatal nodose neurons significantly reduced the size and complexity of the neuronal arbours. Inhibition of IκBα phosphorylation, using either super-repressor IκBα or the pharmacological inhibitor BAY 11-7082, or its subsequent degradation, through the use of the proteosomal inhibitor N-acetyl-Leu-Leu-norleucinal, significantly reduced the total neurite length, total branch number and branching with distance from the cell body (Sholl analysis). Similar, significant, decreases in these parameters were also seen when NFκB-dependent gene transcription was blocked using κB decoy DNA. These effects are not a consequence of reduced neuronal viability, as in each study we confirmed that there was no effect of treatment on the survival of neurons cultured in the presence of BDNF. Additional studies in our laboratory have also indicated that NFκB is not limited to regulating neurite outgrowth in the peripheral nervous system, but also regulates dendritic growth and morphology in layer 2-3 pyramidal neurons in the postnatal mouse somatosensory cortex. Here, both super-repressor IκBα and κB decoy DNA cause significant decreases in overall dendritic length, total number of branch points and branching with distance from the cell body in slice cultures established from P3-P4 mice (Gutierrez et al., 2005). NFκB has also been linked with neurite outgrowth in PC12 cells, where inhibition of NFκB decreases the proportion of cells possessing neurites following Trk A activation (Foehr et al., 2000), although this effect could have represented a downstream consequence of affecting TrkA-induced differentiation, rather than a direct effect on neurite outgrowth.

Initial investigation of upstream activators of NFκB suggested that neither IKKα nor NIK are involved in activating NFκB in cultured nodose neurons. IKKα traditionally forms a complex with IKKβ and NEMO that phosphorylates IκBα and targets it for degradation. Whether IKKα actually plays a role in the activation of NFκB through its classical activation pathway is not certain, as IKKβ has been shown to be

sufficient and essential in such activation (Hayden and Ghosh, 2004). Any involvement of IKKα in the classical NFκB pathway appears to be nuclear where it phosphorylates histone H3 to facilitate NFκB dependent gene transcription (Anest et al., 2003; Birbach et al., 2002; Yamamoto et al., 2003). IKKα, activated by NIK, also targets p100 for degradation, releasing active p52-RelB dimers (Solan et al., 2002; Xiao et al., 2001; Yilmaz et al., 2003). My results therefore suggest that the NFκB activation involved in neurite outgrowth is unlikely to be through the non-canonical pathway. Further investigation of the classical proteins activating IκB degradation and NFκB release would therefore be of interest. Neurite outgrowth cannot be investigated in IKKβ$^{-/-}$ mice as, like p65 knockout mice, they die *in utero* around E15 (Li et al., 1999b; Tanaka et al., 1999). In future experiments I intend to investigate the effects of overexpressing both wild-type and mutant forms of both IKKα and IKKβ.

Analysis of the role of NFκB throughout the period during which nodose neurons extend and elaborate their neuronal processes showed that inhibition of NFκB signalling, using super-repressor IκBα or p65 knockout mice, only altered neuronal morphology during a restricted period of development, starting after E16 and ending before P3. We do not know the cause of this developmental restriction, but it is worth noting that other developmentally restricted changes occur in nodose neurons over this time period, including the expression of Reg-2 mRNA between E18 and P1 described in chapter 4. In terms of neurite outgrowth, this timing coincides not with the period when neurons are extending neurites for the first time, but with the latter end of the period of naturally occurring apoptosis when the remaining neurons are establishing and refining their terminal arborisations (Davies and Lumsden, 1984b). This suggests that NFκB modulates neurite outgrowth during the later stages of target field innervation and process refinement and branching, rather than promoting of de novo neurite extension. Similarly, the inhibition of neurite outgrowth in cortical neurons that occurs on blocking NFκB signalling was detected during a period when these neurons are growing rapidly and establishing and refining their functional connections (Gutierrez et al., 2005). Learning and memory in adults is thought to be dependent on long-term synaptic plasticity, that, alongside other changes, includes

remodelling of axon terminal branches and dendritic morphology. Synaptic complexity increases with both development and environmental input in mature animals (Briones et al., 2004b; Briones et al., 2004a; Buonomano and Merzenich, 1998; Camel et al., 1986; Greenough et al., 1986; Jones et al., 1997; Katz and Davies, 1984; Turner and Greenough, 1985) and some of the changes that occur in plasticity in adulthood may recapitulate events that occur during development. As NFκB is already implicated in learning and memory (Albensi and Mattson, 2000; Meffert et al., 2003; Merlo et al., 2002; Yeh et al., 2002) it is possible that its influence on these processes result from its modulatory role in refining neuronal morphology.

In addition to examining neurite outgrowth, I also examined NFκB activation in our culture system, through transfecting neurons with a plasmid expressing GFP under the control of an NFκB promoter. Unexpectedly, I found robust levels of NFκB activation in neurons grown for 24 hours in the absence of trophic support, and that the levels of NFκB activation were unaffected by the presence of BDNF in the culture medium, either in the short- or long-term. I also confirmed that transfection of neurons with super-repressor IκBα significantly reduced NFκB-induced GFP expression. These results suggest that the treatments we used did not interfere directly with the BDNF-activated signalling pathways that promote neurite outgrowth in developing cultured nodose neurons, but that constitutive NFκB activation co-operates with BDNF to promote neurite outgrowth in late embryonic and early postnatal nodose neurons.

The events driving the basal expression of NFκB seen in cultured nodose neurons are unclear. It may be that NFκB is constitutively active in these neurons, or that its activation is secondary to activation of neuronal receptors by molecules other than BDNF in our culture system. Constitutive activation of NFκB in neurons has been described both *in vivo* and *in vitro*. *In vivo*, constitutive activation is detected in both developing and adult neurons throughout the central nervous system (Acarin et al., 2000; Bhakar et al., 2002; Fridmacher et al., 2003; Kaltschmidt et al., 1994; Schmidt-Ullrich et al., 1996; Gabriel et al., 1999), although *in vivo* each neuron will be receiving a variety of inputs that may be activating NFκB, including, neurotrophic

factors, glutamate, and nitric oxide (Burr and Morris, 2002; Guerrini et al., 1995; Meffert et al., 2003; Simpson and Morris, 1999). Constitutive NFκB activation has also been found *in vitro* (Burr and Morris, 2002; Camandola and Mattson, 2000; Glazner et al., 2001; Piccioli et al., 2001) although in some of these cultures NFκB activity may have been induced by the method of maintaining the cells in culture, such as mild depolarisation (Piccioli et al., 2001). In other studies, constitutive NFκB activity has been attributed to spontaneous calcium ion release from IP_3 sensitive intracellular stores although, again, such release could also be stimulated by growth factors or neuropeptides (Glazner et al., 2001). In the culture system used in the studies reported in this thesis, exogenous factors as a source of NFκB activation can be excluded as the cultures were established in defined medium. However, the cultured neurons may themselves release neurotrophic factors or other proteins into the medium, as may contaminating non-neuronal cells (although there are relatively few non-neuronal cells at the age at which the cultures were established). Such proteins could then act in an autocrine or paracrine manner to promote NFκB activity, particularly in the cultures established at high density.

An alternative possibility is that NFκB is activated by the laminin used as a culture substratum. Laminins are a family of extracellular matrix proteins that bind to cell surface receptors of the integrin family and which are involved in cell motility in a range of cell types (Gu et al., 2002; Hintermann and Quaranta, 2004; Hirosaki et al., 2002; Kariya and Miyazaki, 2004; Powell and Kleinman, 1997; Schober et al., 2000) In neurons, laminins are implicated particularly in neurite outgrowth (DeFreitas et al., 1995; Schmidt et al., 1995; Tomaselli et al., 1993; Yong et al., 1988; Rivas et al., 1992; Powell and Kleinman, 1997; Luckenbill-Edds, 1997), as are other related extracellular matrix proteins that signal through integrins, such as osteopontin (Hikita, 2003) and fibronectin (Yong, 1988; Hikita, 2003; Gomez and LeTourneau, 1994; Kuhn, 1995). Some of these proteins, such as fibronectin either promote or inhibit outgrowth, depending on the neuronal type (Yong, 1988; Hikita, 2003; Gomez and LeTourneau, 1994; Kuhn, 1995). In our culture system laminin-1 is used to attach cells to the culture dish and as a substratum that is permissive for neurite outgrowth (Baron-Van Evercooren, 1982; Yong, 1988). Outside the nervous system

some links have been established between laminins and related extracellular matrix proteins, integrins and NFκB activation. In mammary tumour cell lines, laminin-5 promotes NFκB dependent cell survival (Zahir, 2003). Osteopontin, a cell adhesion molecule related to laminin, that also promotes neurite outgrowth in retinal neurons (Hikita, 2003), has recently been shown to mediate cell motility in breast cancer cell lines via PI3K, NIK, IκBα and NFκB (Philip and Kundu, 2003; Das et al, 2003; Rangaswami, 2004). Integrins themselves have been shown to be developmentally regulated in neurons (Bronner-Fraser, 1992) and if laminin was found to be the source of the constitutive activation of NFκB in our culture system, developmentally regulated changes in integrin subunit expression could potentially explain the developmental restriction of the role of NFκB in neurite outgrowth.

To further understand the role of NFκB in promoting neurite outgrowth in nodose neurons the p65 NFκB subunit was overexpressed in neonatal nodose neurons and NFκB activation, neuronal survival and neurite outgrowth examined. This experimental manipulation greatly increased the level of NFκB-induced gene expression 24 hours after transfection, yet did not appear to promote any additional neurite outgrowth in BDNF-treated neurons, suggesting either that at its basal level NFκB activity is maximal for neurite outgrowth or that the observed outgrowth in BDNF-treated neurons is already the most these neurons can grow in 24 hours. Future experiments could investigate this by establishing a dose-response curve for neurite outgrowth in BDNF-exposed neurons, as here we simply used a concentration previously determined to be saturating for cell survival. A concentration of BDNF could then be used that is suboptimal for neurite outgrowth, perhaps in conjunction with caspase inhibitors, or in Bax knockout neurons, to eliminate any possible effects of reducing BDNF concentrations on cell survival. Such a manipulation may then allow us to detect an increase in neurite outgrowth in response to overexpression of the p65 protein.

Interestingly, overexpression of NFκB in neurons in the absence of trophic support was able to promote cell survival to an extent indistinguishable from that of BDNF, showing that while NFκB is not involved in mediating BDNF-promoted neuronal survival, alone it can be sufficient for cell survival. Similar to nodose neurons,

trigeminal sensory neurons overexpressing p65 also survive without neurotrophic factors, as well as those cultured with NGF (Hamanoue et al., 1999). In these neurons, however, it has been shown that while there is basal activation of NFκB, NGF induced a threefold increase in NFκB transcriptional activity via p75. Nevertheless, this suggests that there is a high level of redundancy in the systems capable of promoting neuronal survival, at least in nodose neurons. These results also indicate that the threshold levels of NFκB activation for neurite outgrowth and neuronal survival are different, with higher levels of NFκB activation being required for NFκB to promote survival in nodose neurons, as compared to the levels required for NFκB to have a positive influence on neurite outgrowth.

A number of intrinsic developmental programs establish certain morphological features depending on the neuronal type. In addition to these intrinsic settings, a wide variety of extrinsic signals regulate the growth and morphology of developing neurites, including neurotransmitters, growth factors, extracellular matrix proteins and neuronal guidance molecules. Each of these inputs engages a specific set of intracellular signalling pathways to influence neurite growth. An additional observation made in the course of our experiments was that while overexpression of p65 in nodose neurons was sufficient to promote neuronal survival, robust activation of NFκB is not alone capable of promoting neurite outgrowth, as no outgrowth was seen in p65-transfected neurons in the absence of BDNF. This confirms the earlier conclusion that for optimal neurite outgrowth in cultured nodose neurons at this age, cooperation is required between NFκB and a BDNF-induced signalling pathway. Signalling pathways known to be implicated in neurite outgrowth include Ras-MEK-ERK, PI3-kinase-Akt and calcium/calmodulin kinases (see chapter 1.5.3). Of these, BDNF can activate both Ras-MEK-ERK and PI3-kinase-Akt with Ras-MEK-ERK being particularly associated with mediating neurite outgrowth (Atwal et al., 2000). Additionally MAPK has been found to be implicated in co-operative promotion of neurite outgrowth by FGF-1 and the extracellular matrix component fibronectin in PC12 cells (Choung et al., 2002). Activation of these pathways influences cytoskeletal dynamics to alter axonal and dendritic growth. For example, phosphorylation of microtubule associated proteins and regulation of the activity of

Rho GTPases and their effectors regulate the structure and dynamics of the actin-based cytoskeleton, and have been linked to neurotrophin activated MAP kinase signalling (Fink and Meyer, 2002; Lundquist, 2003; Miller and Kaplan, 2003). Regulation of transcription has also been shown to influence process growth, with the transcription factors CREB and NeuroD being implicated in mediating the effects of calcium/calmodulin kinases on dendritic growth, although the target genes involved are unknown (Gaudilliere et al., 2004; Redmond et al., 2002). Neurotrophin regulation of β-actin mRNA localisation, and consequently protein levels in growth cones has also been implicated in neurite outgrowth (Zhang et al., 1999; Zhang et al., 2001).

Of the wide variety of genes induced by NFκB in various cell types, several cell adhesion molecules and other proteins that influence cell migration can be identified that may be relevant to process extension in neurons (Pahl, 1999). These include NCAM, β1-integrin and Tenascin-C. NCAM is a cell adhesion molecule that is widely expressed on dendrites and axons of a range of neurons throughout the developing brain (Butler et al., 1998; Chung et al., 1991; Fox et al., 1995; Persohn and Schachner, 1990). During development its expression becomes localised to synapses (Butler et al., 1998; Persohn and Schachner, 1990) and its expression is upregulated in response to electrical activity in cultured striatal neurons, an effect that is mediated by NFκB (Simpson and Morris, 2000). NCAM stimulates neurite growth from a number of neuronal types *in vitro* (Skaper et al., 2001) and an NCAM-mimetic peptide has been found to promote synapse formation and remodelling and to affect learning and memory in adult rats (Cambon et al., 2003; Cambon et al., 2004; Dityatev et al., 2004; Hartz et al., 2003). Clustering of NCAM on cultured cerebellar neurons leads to activation of MAP-kinase (Schmid et al., 1999), which may then promote dendritic growth, as it does in cultured hippocampal, retinal and sympathetic neurons (Dimitropoulou and Bixby, 2000; Vaillant et al., 2002; Wu et al., 2001). NCAM acts principally through homophillic interactions with adjacent cells. In our culture system, such interactions may be involved in the outgrowth seen in the neurons cultured at high density (required for transfection using the gene gun) as the neurons interact with one another. However, they are less likely to be

implicated in the outgrowth seen in the low-density cultures, in which pharmacological inhibitors were used or in cultures establish with neurons from transgenic mice, as contact between individual neurons does not occur. Soluble NCAMs do, however, promote neurite outgrowth in culture and can be detected in the extracellular milieu of living cells (Ronn et al., 1998). Thus, it is possible that upregulation of NCAM expression in cells plated at low density could be acting in this manner. Alternatively it has recently been proposed that polysialated NCAM may sensitise neurons to BDNF. It has been proposed that this may be through inducing pre-clustering of receptor dimers, modifying the turnover and inactivation of the receptor, or by directly affecting the ability of the receptor to bind BDNF, thus amplifying the response of the cell to BDNF (Kiss et al., 2001). While such an effect may be important *in* vivo, where neurons are competing for a limited quantity of growth factor, it is less likely that such an effect is important in our culture system, where BDNF is present in excess, particularly as exogenous BDNF can restore normal function in neurons deficient in PSA-NCAM.

β1-integrin is another NFκB-upregulated cell adhesion molecule (Wang et al., 2003). Unlike NCAM, β1-integrin is involved in mediating the interaction of neurons with the extracellular matrix, rather than adjacent cells, where heterodimer of α and β integrins together act as the cell surface receptors for extracellular matrix proteins such as collagen, laminin and fibronectin (Hikita et al., 2003; Previtali et al., 2001). Interaction between laminin and α1β1 and α3β1 integrins expressed on the growth cone of developing neurites promotes the growth of sensory and sympathetic neurites in culture (DeFreitas et al., 1995; Schmidt et al., 1995; Tomaselli et al., 1993) and anti-integrin β1 antibody in combination with anti L1 and anti-N-cadherin can reduce the growth of ciliary ganglion neurons on a monolayer of cultured Schwann cells (Bixby et al., 1988). I described earlier the potential for such an interaction itself to promote NFκB activation. An additional signalling pathway that may be activated to promote neurite outgrowth through integrin signalling is the Ras-MEK-Erk pathway (Perron and Bixby, 1999). A final possibility is for an NFκB-regulated protein that could mediate the promotion of neurite outgrowth is Tenascin-C (Mettouchi et al., 1997), a protein expressed principally by glial cells in both the peripheral and central

nervous system. It is also transiently expressed by subsets of neurons in the embryonic hippocampus and postnatal spinal cord (Ferhat et al., 1996; Zhang et al., 1995b) and is an extracellular matrix component important in regulating neurite outgrowth and guidance (Joester and Faissner, 2001).

Cooperation between NFκB and BDNF-activated signalling could also be possible prior to their ultimate effects on the cytoskeleton. It has been shown that NFκB requires phosphorylation after it has been released from the IκBα complex to be at its most effective in activation gene transcription. This phosphorlyation appears to act through affecting the ability of NFκB to recruit modulatory proteins that cooperate to more efficiently activate transcription. MAP kinase has been shown to regulate NFκB activity in neurons, independently of PI3 kinase and the typical path of NFκB release from the IκB complex. (Cassarino et al., 2000; Zhou et al., 2004a), and might thus be involved in post-release modifications of NFκB. However as NFκB alone is not capable of promoting neurite outgrowth and the 81% drop in NFκB dependent gene transcription induced in the experiments did not elicit a decrease in neurite outgrowth as robust as might be expected if NFκB were the principal player, it seems more likely that it is the consequences of NFκB activated gene transcription that is cooperating with the downstream effects of an alternative pathway, such as MAPK, that is concomitantly promoting neurite outgrowth.

In summary, a novel role for NFκB signalling has been characterised. The experimental findings show that NFκB transcriptional activity promotes the growth and branching of neuritic processes in the developing peripheral nervous system. This finding extends improving our understanding of the molecular mechanisms involved in establishing and refining neuronal connections during development. Additionally this knowledge has potentially important implications for understanding the molecular and cellular basis of the involvement of NFκB in learning and memory.

Chapter 3

The roles of NFκB in neuronal survival and neurite outgrowth are not mutually exclusive

3.1. Introduction

Nuclear factor κB (NFκB) is a ubiquitously expressed transcription factor involved in a variety of cellular processes throughout the body. These processes include innate and adaptive immune responses, stress responses, cell survival, proliferation and differentiation (Baldwin, Jr., 1996; Karin, 1999; Li et al., 2002). In the previous chapter I described a novel role for NFκB in regulating neurite outgrowth in peripheral sensory neurons of the nodose ganglia. Nodose neurons are a well-defined population of peripheral neurons that can be easily established and maintained *in vitro* in the presence of BDNF (Davies et al., 1993). Unlike other populations of peripheral neurons, no link had been described between BDNF-induced neuronal survival and NFκB, making this an ideal system in which to investigate the role NFκB in neurite outgrowth without having to deal with confounding effects of NFκB on neuronal viability (Hamanoue et al., 1999; Maggirwar et al., 1998). In nodose neurons cultured in the presence of BDNF, several methods of inhibiting NFκB signalling caused significant decreases in neurite length and branching, and revealed the importance of NFκB for neurite outgrowth *in vitro*. These results raised the question of whether NFκB has different effects on neurite growth and cell survival in different types of neurons.

The superior cervical ganglion (SCG), part of the sympathetic nervous system, is derived from a different precursor cell population than nodose neurons, and responds to a different set of neurotrophic factors (Le Douarin et al., 2004; Wyatt and Davies, 1995; Zhou and Rush, 1995) reviewed in chapter 1). The survival of these neurons with NGF is dependent in part on phoshotidylinositol-3 kinase (PI3K) and protein kinase C (PKC) and, at the transcriptional level, NFκB (Maggirwar et al., 1998; Pierchala et al., 2004; Tsui-Pierchala et al., 2000). In this chapter I investigate the

separation between NFκB-induced neurite outgrowth and survival and whether in specific cell types NFκB induces exclusively neurite outgrowth or survival, or whether these effects can occur side by side. I do this by examining the consequences of inhibiting NFκB signalling in neonatal SCG neurons. Caspases are able to rescue SCG neurons from NGF deprivation without any observable changes in process morphology (Werth et al., 2000), and were used to prevent cell death as a consequence of inhibiting NFκB signalling. I also investigated whether NFκB is involved in neurite outgrowth in neonatal nodose neurons maintained *in vitro* by neuropoietic cytokines. These proteins are as effective as BDNF at promoting neuronal survival (Chapter 4; (Middleton et al., 2000). Unlike BDNF, however, the survival response of nodose neurons to these cytokines has been reported to be dependent on NFκB signalling (Middleton et al., 2000). In this chapter I report that NFκB regulates neurite length and branching in sympathetic neurons maintained in culture by NGF and caspase inhibitors. It also plays a role in the neurite outgrowth from nodose neurons treated with neuropoietic cytokines. However, this effect varies according to the effectiveness of the individual cytokines at promoting neurite growth. Additionally, in the experimental paradigm used here, the survival of nodose neurons in the presence of cytokines does not appear to be NFκB-dependent. My results suggest that in nodose neurons, NFκB is only involved in promoting neurite outgrowth, but that in sympathetic neurons NFκB promotes both survival and outgrowth.

3.2. Material and Methods

Cultures of nodose neurons were established, transfected and analysed in the manner described in chapter 2. Briefly, P0 mice were killed by carbon dioxide asphyxiation followed by decapitation, and the nodose ganglia dissected. Ganglia were then dissociated by trypsinisation followed by trituration and plated in defined serum-free medium (see appendix) on a polyornithine/laminin substratum in 35mm tissue culture dishes. Transfection was performed using ballistic transfection of gold microcarriers coated with plasmids coding for pYFP and either pSR-IκB-α or a pCDNA control plasmid, and the cultures supplemented with 50ng/ml CNTF or LIF (Chemicon, CA). Neuronal survival was estimated by counting the number of neurons labelled with YFP surviving 48 hours after transfection and expressing this as a percentage of the number of labelled neurons counted 12 hours after transfection. Each cell was also examined using phase-contrast conditions to corroborate cell survival. At 24 and 48 hours after transfection, neurons were digitally imaged using confocal microscopy and neurite outgrowth quantified using Sholl analysis (Sholl, 1953), and for total neurite length and number of branch points.

SCG neurons were cultured in a similar manner to that of nodose neurons. The SCG is located adjacent to the nodose ganglia in embryonic and early postnatal mice therefore the dissection is identical until the last step when the SCG, rather than the nodose ganglia, is removed. The location of the SCG can be seen in figure 2.1. Trypsinisation and trituration was identical to that of the nodose ganglia. For the cultures where the pharmacological inhibitor BAY 11-7082 was used the neurons were plated on the same polyornithine/laminin substratum in 35mm dishes, however cultures of neurons that were to be treated with the NFκB inhibitory peptide SN50 were established in dishes 35mm dishes containing four 11mm wells, in a total volume of 80μl, to minimise the quantity of the peptide required. All SCG cultures were performed at low cell densities and supplemented with 10ng/ml NGF.

Investigating the potential role of NFκB signalling in regulating the growth of sympathetic neurons was complicated by the fact that NFκB is involved in mediating the survival-promoting effects of NGF (Maggirwar et al., 1998). This would mean that if neurons were cultured with NGF and NFκB inhibitor alone any effect on neurite outgrowth could be attributed to the loss of a specific subpopulation of NFκB dependent neurons. To circumvent this effect, cultures were treated with the caspase inhibitor Boc-D-FMK (Caspase inhibitor III, Calbiochem, UK) at a concentration of 250μm. Neurotrophic factors (NGF, 10ng/ml) were added after an initial cell count and a 45min pre-incubation with the caspase inhibitor plus either BAY 11-7082 (10μM) or SN50 (25μg/ml). Survival and neurite outgrowth were quantified as described in chapter 2. All statistical comparisons are performed using ANOVA followed by Fishers' post-hoc test.

3.3. Results

The results in the previous chapter showed that NFκB is important in regulating the growth and morphology of nodose neurons cultured in the presence of BDNF. In this chapter I performed further studies to investigate whether this role was maintained in neurons in which NFκB is also required for cell survival.

3.3.1. NFκB signalling is important to the neurite outgrowth of neonatal sympathetic neurons

The aim of this first investigation was to study whether NFκB is involved in neurite outgrowth in the sympathetic neurons of the superior cervical ganglion (SCG), a population of peripheral neurons in which NFκB has previously been shown to mediate neurotrophin-induced neuronal survival (Maggirwar et al., 1998). Neonatal SCG neurons were cultured in the presence of NGF and treated with either BAY 11-7082, an inhibitor of IκB-α phosphorylation, or SN50. SN50 is synthetic peptide that contains the nuclear localisation sequence of p50, thus inhibiting the translocation of active NFκB dimers to the nucleus. SN50 has previously been shown to be effective in preventing NFκB-induced gene transcription in sympathetic neurons, and consequently to decrease the survival response of SCG neurons to NGF (Maggirwar et al., 1998). Neurons were imaged using a confocal microscope after a 24h incubation and neurite outgrowth assessed by Sholl analysis and measurement of total neurite length and branching. The potential confounding effect of NFκB on neuronal survival was controlled by also treating cultures with caspase inhibitor III, an irreversible broad-spectrum caspase inhibitor, which is capable of rescuing neurons from death induced by neurotrophin withdrawal (Werth et al., 2000).

3.3.1.1. BAY 11-7082 reduces the NGF-induced neurite outgrowth of SCG neurons maintained in vitro *in the presence of caspase inhibitors*

P0 SCG neurons were grown in low-density dissociated cultures in defined medium supplemented with NGF (10ng/ml) and caspase inhibitor III (250μM), and half the

culture dishes were treated with BAY 11-7082 (10μM). Figure 3.1A shows the survival of these neurons after 24 hours in culture. Sixty percent of SCG neurons cultured in medium supplemented with NGF alone were surviving after 24 hours, while in unsupplemented medium less that 5% of the neurons remained. Caspase inhibitor III, in the absence of any trophic support, rescued the same proportion of SCG neurons that were supported by the presence of NGF alone, as there is no significant difference between the survival between these two groups ($p>0.5$) and no additive effect of treating SCG neurons with both NGF and caspase inhibitor III. The addition of BAY 11-7082 to neurons cultured in the presence of NGF killed 81% of neurons and these were also effectively rescued by the addition of caspase inhibitor III to the culture medium. These results confirm that NGF-induced neuronal survival is NFκB dependent, as treating NGF-supported neurons with the IκB-α phosphorylation inhibitor BAY 11-7082 resulted in a significant amount of cell death. As caspase inhibitor rescued all neurons in the BAY 11-7082 treated cultures, neurite outgrowth in BAY 11-7082-treated neurons was studied independently of the effect of this compound on neuronal survival.

Neurite outgrowth was quantified in images of neurons captured after 24 hours in culture. The resultant Sholl analysis can be seen in Figure 3.1B. Neurons cultured in medium supplemented with NGF alone extended large neurite arbours, which produced a Sholl analysis similar in shape to the equivalent culture of nodose neurons, with peak of 7.7 intersections at 210μm from the cell soma. Interestingly addition of caspase inhibitor III to these neurons produced a significant decrease in the number of intersections at each ring between 90 and 450μm from the cell body

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* **Figure 3.1** *BAY 11-7082 reduces neurite outgrowth from SCG neurons*
P0 SCG neurons were incubated in medium containing 100μM caspase inhibitor III, 10ng/ml NGF and either the IκB-α phosphorylation inhibitor BAY 11-7082 at a concentration of 10nM or vehicle control. Vehicle treated neurons were also grown without NGF. A) Percent survival after 48h in culture. B) Sholl analysis of neurite arbor morphology after 24h in culture. C) Total neurite length after 24h in culture. D) Number of branch points in neurite arbors after 24h in culture. E) Photomicrographs of a typical β-III tubulin stained, vehicle or BAY 11-7082 treated neurons grown for 24 hours with NGF and caspase inhibitor III (Scale bar = 50 μm). The means and standard errors of data obtained from 60-90 neurons in each experimental condition shown. Statistical comparisons shown are with respect to the control transfected neurons, * $p<0.05$, ** $p<0.001$.

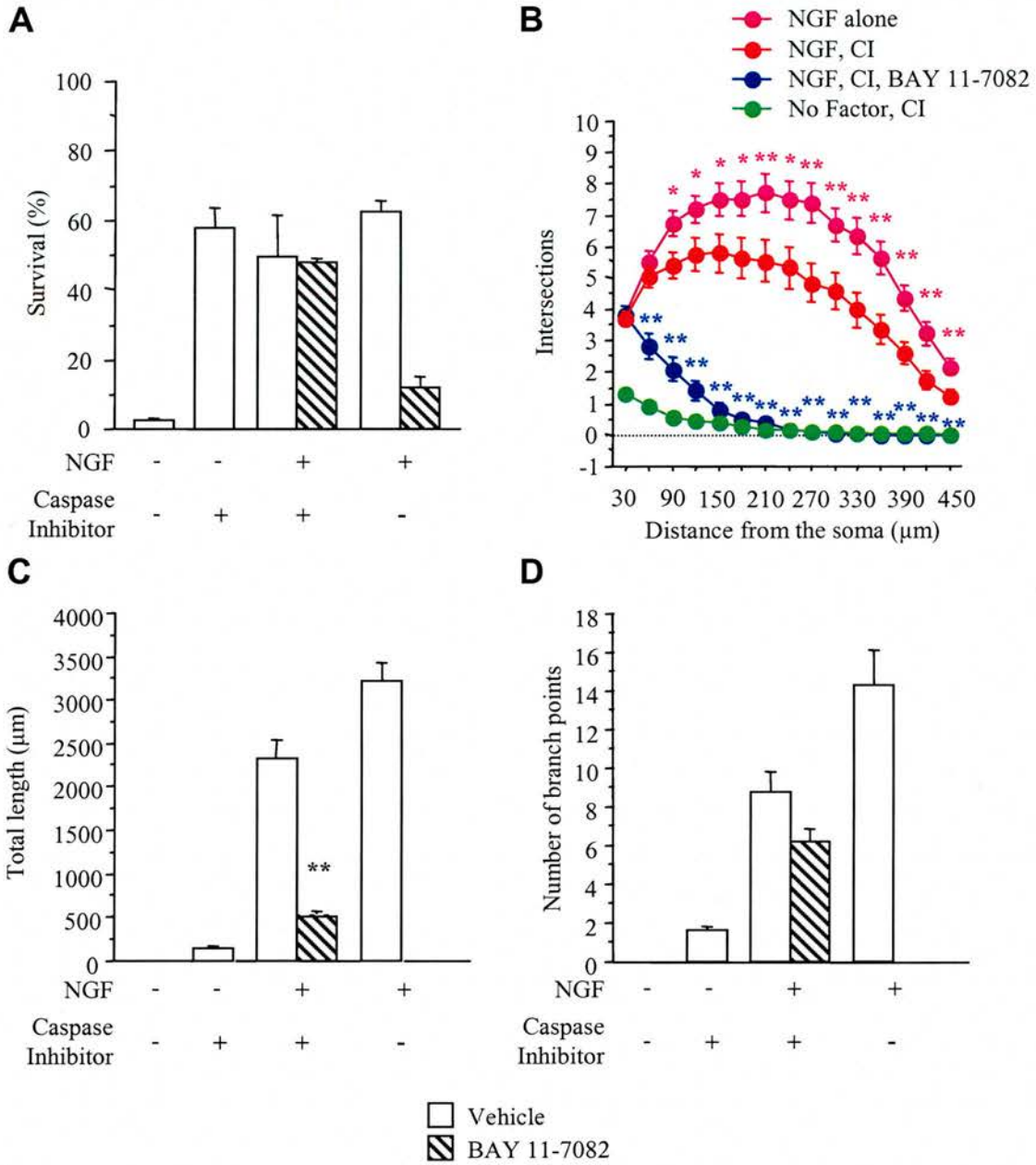
A
Survival (%)
100
80
60
40
20
0
NGF - - + +
Caspase Inhibitor - + + -
B
NGF alone
NGF, CI
NGF, CI, BAY 11-7082
No Factor, CI
Intersections
Distance from the soma (µm)
30 90 150 210 270 330 390 450
C
Total length (µm)
4000
3500
3000
2500
2000
1500
1000
500
0
**
NGF - - + +
Caspase Inhibitor - + + -
D
Number of branch points
18
16
14
12
10
8
6
4
2
0
NGF - - + +
Caspase Inhibitor - + + -
Vehicle
BAY 11-7082
E
Control
BAY 11-7082

Figure 3.1

($p<0.05$ for rings 90-180; $p<0.001$ for rings 210-450). This effect could represent a direct involvement of caspases in neurite outgrowth or could be due to the neurons, whilst surviving, not being as healthy as neurons grown in the presence of NGF alone. Most important, however, is the observation that neurons grown with NGF plus caspase inhibitor and treated with BAY 11-7082 exhibited a highly significant decrease in neurite outgrowth compared to neurons grown with NGF plus caspase inhibitor alone. The number of intersections per ring, in the vehicle-treated group, increased from 3.7 intersections at 30μm to 5.8 intersections at 150μm from the cell body, subsequently decreasing slowly towards 450μm. In neurons treated with BAY 11-7082, the initial number of intersections was very similar, starting at 3.8 intersections at 30μm, but there was no following peak, with the mean number of intersections tailing off rapidly until 150μm, where there was less than 1 intersection per ring ($p<0.001$ from the 60μm ring onwards). The only distinction between this BAY 11-7082-treated group of neurons and those grown with caspase inhibitors alone appears to be that they extended more initial neurites. Typical neurons from the NGF + caspase inhibitor III and the NGF + caspase inhibitor + BAY 11-7082 groups can be seen in figure 3.1E. The effects of treatment with caspase inhibitors and BAY 11-7082 seen in the Sholl analysis are also reflected in the quantification of total neurite length, where addition of caspase inhibitors to NGF-treated neurons caused a 28% decrease in total neurite length and a further addition of BAY 11-7082 resulted in an additional decrease in length of 56%. Interestingly, while addition of caspase inhibitors to NGF-treated neurons also resulted in a 39% decrease in neurite branching, the further addition of BAY 11-7082 produced only an additional 18% decrease in neurite outgrowth, an effect that was not significantly different from that of adding only the caspase inhibitor to NGF-treated neurons ($p>0.1$), suggesting that in SCG neurons NFκB may only be involved in signalling neurite growth, and not in neurite branching. Nevertheless, these results suggest that the phosphorylation of IκB-α is important for NGF-promoted neurite outgrowth in neonatal SCG neurons.

3.3.1.2. SN50 reduces the NGF-promoted outgrowth of SCG neurons grown in the presence of caspase inhibitors

The role of NFκB in the neurite outgrowth of SCG neurons was confirmed using the inhibitory peptide SN50, previously used by (Maggirwar et al., 1998) to interfere with NFκB-dependent neuronal survival. Neurons were cultured in the same manner as in the previous study, with different combinations of NGF (10ng/ml), caspase inhibitor III (250μM) and SN50 (25μg/ml) or its inactive control peptide, SN50M, at the same concentration. SN50 has been reported to completely block NGF induced neuronal survival in SCG neurons at 100μg/ml, with this effect disappearing by the time this concentration is reduced to 50μg/ml (Maggirwar et al., 1998). In our hands SCG neurons proved more sensitive to SN50, with maximal cell death being observed at a concentration of 50μg/ml (data not shown) and significant cell death (50.3%, $p<0.001$) still being detected at dose of 25μg/ml (figure 3.2A). It was this lower concentration that was used in the analysis of neurite outgrowth, as 250μM caspase inhibitor III was unable to completely reverse the cell death induced by 50μg/ml SN50 (data not shown).

Neurite outgrowth was analysed in images of SCG neurons taken 24 hours after the addition of trophic support. Sholl analysis revealed that neurons cultured in the presence of NGF, caspase inhibitor III and SN50 had less intersections with each ring between 60μm and 450μm from the cell body, as compared to neurons treated with NGF, caspase inhibitor III and the control peptide. This difference reached significance at 180μm from the cell soma ($p<0.05$) and between 240 and 450μm from the cell soma (figure 3.2B). This effect could also be seen in the analysis of

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* **Figure 3.2** *SN50 reduces neurite outgrowth from SCG neurons*
P0 nodose neurons were incubated in medium containing 100μM caspase inhibitor III, 10ng/ml NGF and either the NFκB inhibitory peptide SN50 at a concentration of 25μg/ml or a peptide control (SN50M). Control treated neurons were also grown without NGF. A) Percent survival after 48h in culture. B) Sholl analysis of neurite arbor morphology after 24h in culture. C) Total neurite length after 24h in culture. D) Number of branch points in neurite arbors after 24h in culture. E) Photomicrographs of a typical β-III tubulin stained, control or SN50 treated neurons grown for 24h with NGF (Scale bar = 50 μm). The means and standard errors of data obtained from 60-90 neurons in each experimental condition shown. Statistical comparisons shown are with respect to the control transfected neurons, * $p<0.05$, ** $p<0.001$.

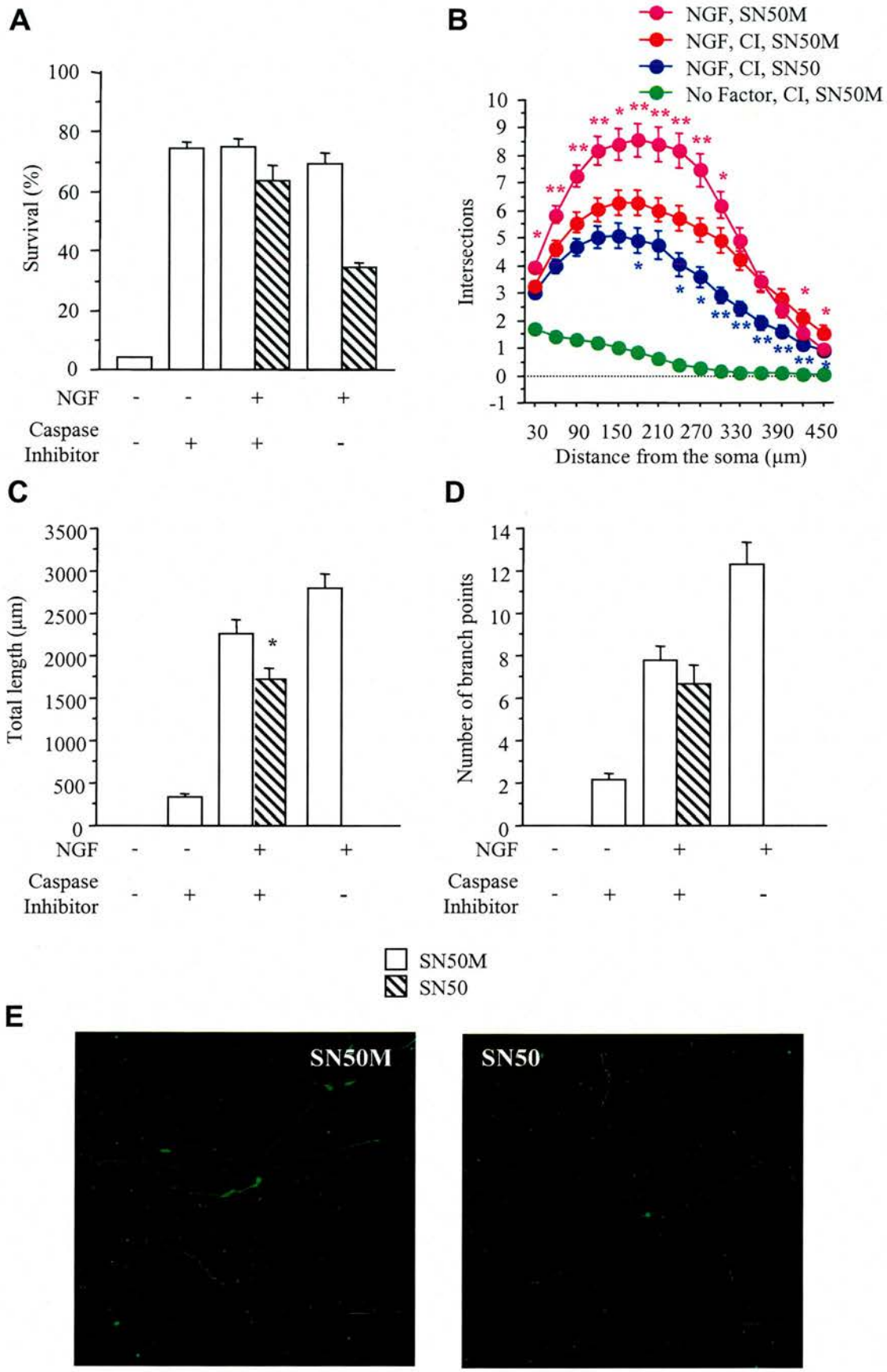

A
Survival (%)
100
80
60
40
20
0
NGF - - + +
Caspase Inhibitor - + + -
B
NGF, SN50M
NGF, CI, SN50M
NGF, CI, SN50
No Factor, CI, SN50M
Intersections
Distance from the soma (μm)
30 90 150 210 270 330 390 450
C
Total length (μm)
3500
3000
2500
2000
1500
1000
500
0
NGF - - + +
Caspase Inhibitor - + + -
D
Number of branch points
14
12
10
8
6
4
2
0
NGF - - + +
Caspase Inhibitor - + + -
SN50M
SN50
E
SN50M
SN50

Figure 3.2

total neurite length, where SN50 induced a 19% larger decrease in total neurite length than that observed in neurons grown with NGF, caspase inhibitor III and treated with the control peptide SN50M ($p<0.05$) (figure 3.2C). As with BAY 11-7082, there was not a significant difference between the decrease in number of branch points seen with neurons supported by NGF and caspase inhibitor III and treated with SN50 (46%) and neurons grown in the same conditions but treated with SN50M (37%) (Figure 3.2D). Typical neurons from the NGF + caspase inhibitor III + SN50M and the NGF + caspase inhibitor + SN50 groups are shown in figure 3.2E. Taken together with the data shown in figure 3.1, these results suggest NFκB-activated gene transcription is important in promoting extension, but not branching of neurites in SCG neurons.

3.3.2. CNTF, but not LIF, promotes the outgrowth of neonatal nodose neurons through NFκB-activated gene transcription

The second group of investigations addressed the question of whether NFκB is involved in the outgrowth of neonatal nodose neurons maintained *in vitro* by neuropoietic cytokines. This was done by transfecting neonatal nodose neurons with the super-repressor IκB-α construct, culturing them in the presence of either CNTF or LIF and analysing outgrowth and survival.

3.3.2.1. Super-repressor IκB-α reduces CNTF-induced neurite outgrowth in nodose neurons

Dissociated cultures of P0 nodose neurons were established and cultured for three hours in the absence of neurotrophic factors until they had attached to the culture dish. Using the Helios gene gun, neurons were transfected with pYFP in combination with either the super-repressor IκB-α construct or an empty control plasmid. At this point CNTF (50ng/ml) was added and the cultures were incubated at 37°C for 24 hours before the neurons were imaged for analysis of neuronal morphology. A further set of images was also taken at 48 hours to assess the morphology of the neurons at this later time point. Neuronal survival was assessed by comparing the number of YFP-positive neurons surviving at 48 hours to the number of neurons expressing YFP at 12 hours after transfection. Figure 3.3A shows that 59% of the

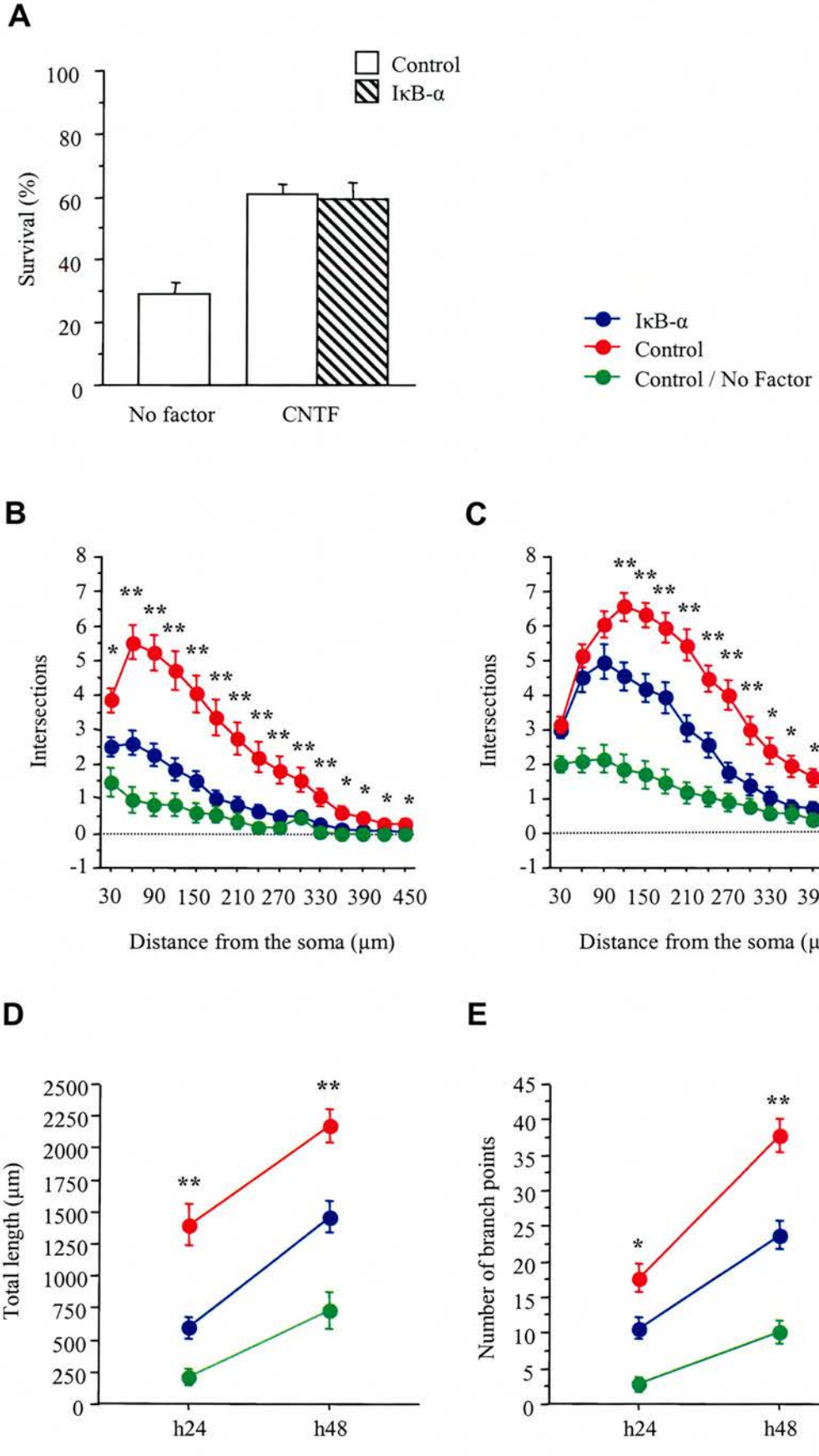
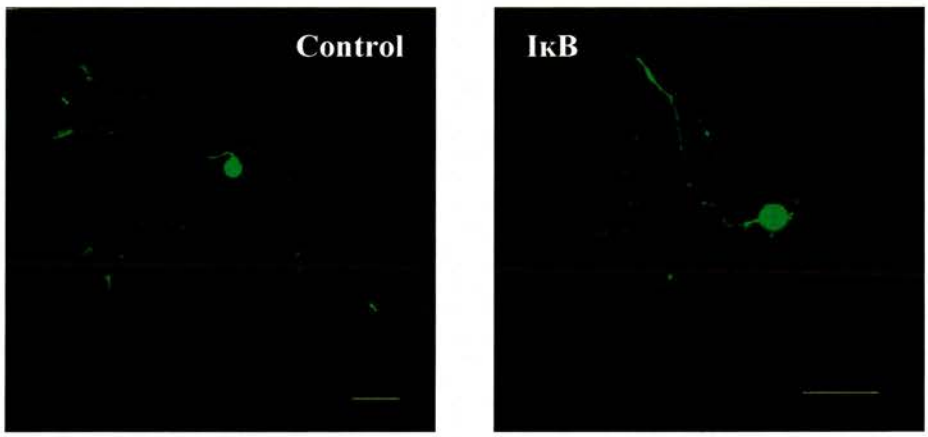

A
Control
IκB-α
Survival (%)
100
80
60
40
20
0
No factor
CNTF
IκB-α
Control
Control / No Factor
B
Intersections
Distance from the soma (μm)
30 90 150 210 270 330 390 450
C
Intersections
Distance from the soma (μm)
30 90 150 210 270 330 390 450
D
Total length (μm)
2500
2250
2000
1750
1500
1250
1000
750
500
250
0
h24
h48
E
Number of branch points
45
40
35
30
25
20
15
10
5
0
h24
h48

Figure 3.3

F



G

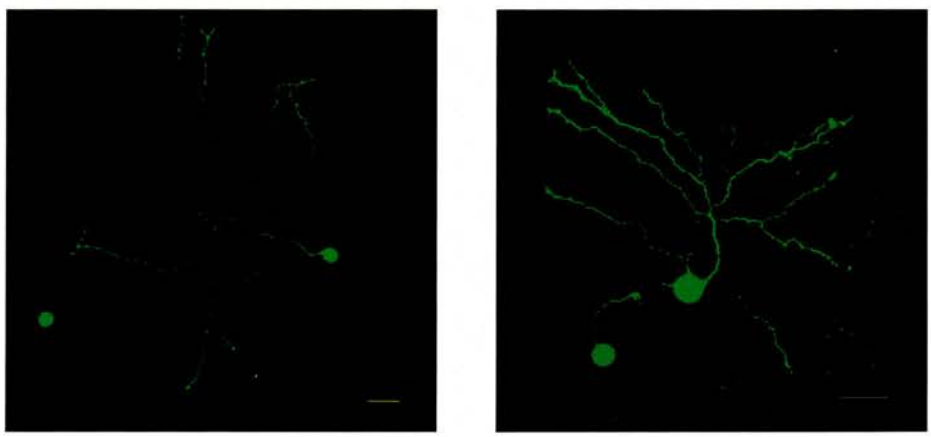


Figure 3.3 *Super-repressor IκB-α reduces neurite growth from neonatal nodose neurons cultured in the presence of CNTF, but does not affect survival*
P0 nodose neurons were transfected with a YFP expression plasmid together with either a super-repressor IκB-α plasmid or an empty control plasmid and incubated in medium containing 50 ng/ml CNTF. Control transfected neurons were also grown without CNTF. A) Percent survival 48 hours after transfection. B) Sholl analysis of neurite arbor morphology 24 hours after transfection. C) Sholl analysis of neurite arbor morphology 48 hours after transfection. D) Total neurite length 24 and 48 hours after transfection. E) Number of branch points in neurite arbors 24 and 48 hours after transfection. F) Photomicrographs of a typical control and super-repressor IκB-α transfected neurons grown for 24 and 48 hours with CNTF (Scale bar = 50 μm). The means and standard errors of data obtained from 60-90 neurons in each experimental condition are shown. Statistical comparisons shown are with respect to the control transfected neurons, * $p<0.05$, ** $p<0.001$.

neurons transfected with IκB-α and cultured for 48 hours in the presence of CNTF survived, which was as good as the survival of control transfected neurons (61%), and this survival was significantly above that of control-transfected neurons cultured in the absence of neurotrophic support (29%; $p<0.001$). This suggests that, in this culture system, blocking NFκB activation by using super-repressor IκB-α has no effect on neuronal survival.

Neuronal morphology was analysed by Sholl analysis and by measuring total neurite length and the number of branch points for each neuron. Figures 3.3B and C show the Sholl analysis of neuronal morphology at 24 and 48 hours respectively. An additional analysis of neurite length was performed at 48 hours as the overall neurite arbours in these cultures appeared smaller at 24 hours than those of the cultures shown in chapter 2, where neurons were grown in the presence of BDNF. P0 control-transfected neurons cultured for 24 hours in the presence of BDNF had a maximum no of intersections of 14.2 at a mean distance of 150μm from the cell body, whereas in neurons cultured for 24 hours in the presence of CNTF the maximum number of intersections in control-transfected neurons was only 5.5 and this occurred at 60μm. At 48 hours this had increased to 6.6 intersections at the ring 120μm from the cell soma. However this still did not approach the outgrowth seen in BDNF-treated neurons at 24 hours, suggesting that CNTF is less effective than BDNF at promoting neurite outgrowth, despite promoting similar effects on neuronal survival. At both 24 and 48 hours after transfection, the neurite outgrowth seen in neurons transfected with super-repressor IκB-α was significantly decreased compared to control transfected neurons. At 24 hours the number of intersections per ring barely increased after the first ring, and was half that seen in control neurons (2.6 intersections at 60μm; $p<0.001$). At 48 hours a peak could be seen, but this is at 90 rather than 120μm from the cell body and only 4.6 intersections were counted at the maximum, compared to 6.6 in control neurons. These differences are reflected in the analysis of total neurite length, where 57% and 33% decreases were seen at 24 and 48h respectively ($p<0.001$; figure 3.3D). Likewise, there were 40% and 37% decreases in branches detected at 24 and 48h respectively ($p<0.05$ and $p<0.001$; figure 3.3E). At both time points, however, the length and branching seen in IκB-α

transfected neurons was higher than that seen in control-transfected neurons cultured in the absence of trophic support. Typical control and IκB-α transfected neurons cultured in the presence of CNTF are shown in figures 3.3F and G (24 and 48h respectively). Overall these results suggest that while CNTF is less effective than BDNF at promoting neurite outgrowth in neonatal nodose neurons, there is a similar requirement for NFκB signalling to promote neurite outgrowth when nodose neurons are cultured in the presence of CNTF.

3.3.2.2. Super-repressor IκB-α does not effect the neurite outgrowth of nodose neurons cultured in the presence of LIF

To assess whether NFκB is also necessary to promote neurite outgrowth in neurons cultured in the presence of LIF, dissociated cultures of P0 nodose neurons were established and transfected in the same manner as that described for CNTF. Neuronal survival was assessed after 48 hours in culture with LIF, where transfection with IκB-α had no effect of neuronal survival compared to control-transfected neurons (figure 3.4A). Neuronal morphology was assessed at 24, 48 and 72 hours after transfection and it was found that LIF is a poor promoter of neurite outgrowth, even when compared to the growth promoting ability of CNTF. Analysis of total neurite length and number of branch points showed that by 48 hours *in vitro*, neurites of

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* **Figure 3.4** *Super-repressor IκB-does not affect the survival or neurite growth from neonatal nodose neurons cultured in the presence of LIF*
P0 nodose neurons were transfected with a YFP expression plasmid together with either a super-repressor IκB-α plasmid or an empty control plasmid and incubated in medium containing 50 ng/ml LIF. Control transfected neurons were also grown without LIF. A) Percent survival 48 hours after transfection. B) Total neurite length up to 72 hours after transfection. C) Number of branch points in neurite arbors up to 72 hours after transfection. D) Sholl analysis of neurite arbor morphology 24 hours after transfection. E) Sholl analysis of neurite arbor morphology 48 hours after transfection. F) Sholl analysis of neurite arbor morphology 72 hours after transfection. G) Photomicrographs of a typical control and super-repressor IκB-α transfected neurons grown for 24, 48 and 72 hours with LIF (Scale bar = 50 μm). The means and standard errors of data obtained from 60-90 neurons in each experimental condition are shown. Statistical comparisons shown are with respect to the control transfected neurons, * $p<0.05$, ** $p<0.001$.

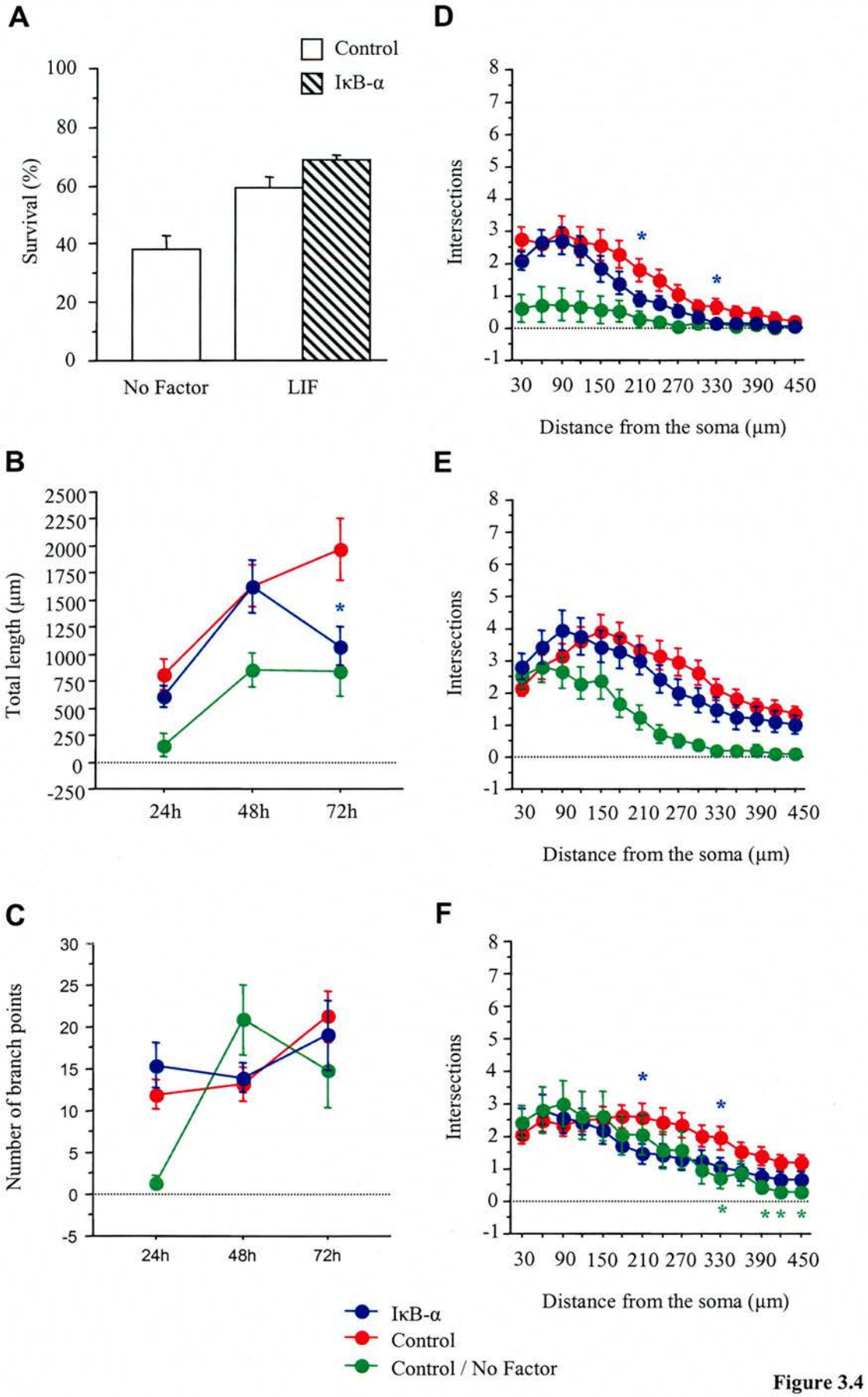
A
Control
IκB-α
Survival (%)
100
80
60
40
20
0
No Factor
LIF
D
Intersections
8
7
6
5
4
3
2
1
0
-1
30 90 150 210 270 330 390 450
Distance from the soma (μm)
B
Total length (μm)
2500
2250
2000
1750
1500
1250
1000
750
500
250
0
-250
24h
48h
72h
E
Intersections
Distance from the soma (μm)
C
Number of branch points
30
25
20
15
10
5
0
-5
24h
48h
72h
F
Intersections
Distance from the soma (μm)
IκB-α
Control
Control / No Factor

Figure 3.4

neurons cultured in the presence of CNTF were 30% longer and possessed 187% more branch points than neurons cultured with LIF (compare figures 3.3D and E with 3.4B and C). By Sholl analysis, control neurons cultured in the presence of LIF showed a peak number of intersections (3.0) at 90μm from the cell body at 24 hours (fig 3.4D), which increased to only 3.9 intersections at 150μm 48 hours after transfection (figure 3.4E). By 72 hours after transfection the maximal number of intersections had decreased, to 2.6 intersections, although this peak was further from the nucleus, at 180μm, and a significant difference in number of intersections between the control transfected neurons cultured in the presence of LIF and the control-transfected neurons cultured in the absence of neurotrophic support was only achieved at 330, 390, 420 and 450μm from the cell body ($p<0.05$; figure 3.4F). In neurons transfected with IκB-α there was a tendency towards a decrease in the number of intersections, however this only reached significance at two distances (210 and 330, $p<0.05$) at 24 hours after transfection and two distances (210 and 330μm, $p<0.05$) 72 hours after transfection. Transfection of LIF-treated neurons with the super-repressor IκB-α plasmid caused a significant decrease in total neurite length only at 72 hours after transfection (46%; $p<0.05$) and has no significant effect on the number of branch points at any time point (Figure 3.4B and C). Overall these results suggest that LIF is ineffective at promoting neurite outgrowth in neonatal nodose neurons, as compared to either CNTF or LIF, and that transfection with super-repressor IκB-α has little, if any, effect on the small outgrowth seen.

3.4. Discussion

This study investigated whether the two roles of NFκB previously identified in neurons, survival (Maggirwar et al., 1998; Middleton et al., 2000) and neurite outgrowth (chapter 2), are mutually exclusive or whether, in some circumstances, NFκB is capable of influencing both neuronal survival and neurite outgrowth simultaneously. This was done by investigating the consequences of inhibiting NFκB activation in nodose neurons cultured in the presence of neuropoietic cytokines and in SCG neurons sustained *in vitro* by NGF.

In neonatal nodose neurons, both CNTF and LIF effectively promoted neuronal survival, but that LIF was a much poorer promoter of neurite outgrowth, as compared to CNTF. After 48 hours *in vitro*, neurites of neurons cultured in the presence of CNTF were 30% longer and possessed a massive 187% more branch points than neurons cultured with LIF, with this decreased branching meaning that in the Sholl analysis the growth of LIF-treated neurons was barely above that of neurons cultured without neurotrophic support. Generally, in the nervous system, there are few reported differences between either the *in vitro* or *in vivo* effects of CNTF and LIF (Turnley and Bartlett, 2000), an impression substantiated by the considerable redundancy between neuropoietic cytokines revealed by comparison of ligand and receptor knockout animals (DeChiara et al., 1995; Li et al., 1995; Masu et al., 1993; Ware et al., 1995). One interesting difference that is observed concerns differentiation. Some cell types release CNTF in combination with soluble CNTFRα, allowing CNTF to act on cells that do not themselves express CNTFRα (Elson et al., 2000; Marz et al., 1999). Marz et al. (2002), have produced a CNTF-sCNTFRα fusion protein (hyper-CNTF) and compared its effects to those of LIF in several cells lines, finding that in PC12 cells hyper-CNTF, but not LIF, induced differentiation into a neuronal phenotype. They also observed that while both LIF and hyper-CNTF induced STAT-3 activation in this cell line, only hyper-CNTF was capable of activating MAP kinase. This agrees with earlier studies that have shown the ability of neuropoietic cytokines to induce neuronal differentiation or gliogenesis in neural precursors depends upon a balance between the activation of the MAP kinase and

JAK-STAT signalling pathways, both of which are initiated by activation of the LIF receptorβ/gp130 receptor complex (Bonni et al., 1997; Ihara et al., 1997). The presence of other neurotrophic factors, including NGF, can alter the final phenotype of neural precursor cells *in vitro*. This effect can be brought about by activation of MAP kinase pathways or by inducing the activation of SOCS (Suppressor of Cytokine Signalling) proteins that are capable of inhibiting JAK-STAT signalling (Ihara et al., 1997; Turnley and Bartlett, 2000) (Li and Stark, 2002). If the balance swings towards more MAP kinase activation a neuronal phenotype, and consequently neurite outgrowth, is observed (Ihara et al., 1997; Turnley and Bartlett, 2000).

Both CNTF and LIF are capable of promoting neurite outgrowth. However, the presence of other factors or cells can influence the ability of these factors to promote neurite outgrowth and survival, and can even cause neuropoetic cytokines of have an inhibitory effect on the growth of some processes (Burnham et al., 1994; Edoff and Jerregard, 2002; Gillespie et al., 2001; Goldberg et al., 2002; Guo et al., 1997; Guo et al., 1999; Kessler et al., 1993; Ng et al., 2003; Richards et al., 1992). Such retractory effects can be important in establishing neuronal morphology (Guo et al., 1999). Guo et al (1999) found that LIF and CNTF inhibited dendritic formation and caused dendritic retraction in sympathetic neurons cultured with BMP-7 and NGF. Ng et al (2003) found that LIF and CNTF, when used in combination with NGF, in either PC12 cells or sympathetic neurons decreases NGF-induced neurite outgrowth through inhibition of Rac1 activation (Ng et al., 2003). Thus a similar change in the balance between the JAK-STAT and MAP kinase signalling to that which alters the effect of cytokines on differentiation may explain the switch between neuropoietic cytokines promoting or inhibiting neurite outgrowth in different circumstances. A difference in the abilities of CNTF and LIF to activate MAP kinase signalling in neonatal nodose neurons, similar to that described by (Marz et al., 2002) in PC12 cells, could explain the different outgrowth responses seen in my experiments.

In neonatal nodose neurons supported *in vitro* by CNTF I found that transfection with the super-repressor IκBα plasmid had no effect on survival, but caused a significant decrease in neurite outgrowth. In neurons maintained by the presence of LIF, transfection with super-repressor IκBα had no effect on survival, but unlike

neurons sustained with CNTF, no effect on neurite outgrowth was not found. It is possible that an effect on neurite growth was undetectable in LIF-supplemented cultures because the low level of neurite growth promoted by this factor. Alternatively, the additional neurite outgrowth seen in CNTF-stimulated neurons could be via an NFκB-dependent pathway not activated by LIF.

While BDNF does not activate NFκB-induced gene transcription, it has been shown that cytokines can activate NFκB (Middleton et al., 2000; Nishimune et al., 2000). Nishimune et al. (2000) found that overexpression of IκBα abrogated the CNTF-induced survival response in motoneurons, while Middleton et al. (2000) found that microinjection of a construct coding for super-repressor IκBα into E18 nodose neurons prevented both CNTF- and LIF-induced neuronal survival. Using particle-mediated gene transfection in neonatal neurons, we could not confirm the effect of transfecting nodose neurons with pSR-IκBα, reported in Middleton et al. (2000), on neuronal survival in the presence of CNTF, although the observed effect on neurite outgrowth confirms that our plasmid was functional. The culture systems used were very similar, however there are a few possibilities to explain this difference. One possibility is that the sensitivity of CNTF-supported nodose neurons to inhibition of NFκB activation is lost between E18 and P0. Alternatively, the high density cultures, used for ballistic transfection could potentially have affected the need for functional NFκB signalling to promote neuronal survival. If this were true, however, one might expect culturing neurons at high density alone might be sufficient to promote significant neuronal survival, which is not the case.

In agreement with Maggirwar et al. (1998), I found that NFκB is important for NGF-induced neuronal survival of SCG neurons. Furthermore, the IκB-phosphorylation inhibitor BAY 11-7082 and the inhibitory peptide SN50 also caused significant decreases neurite outgrowth in SCG neurons whose survival was sustained by caspase inhibitors. This suggests that whereas NFκB regulated the survival and neurite growth of SCG neurons, it appears only to be important for regulation of neurite growth in sensory neurons of the nodose ganglia.

Although NFκB plays a role in regulating neurite outgrowth in both sympathetic and sensory neurons, the effects of inhibiting NFκB in these two types of neurons are qualitatively and quantitatively different. While interfering with NFκB signalling caused decreases in both total neurite length and the number of branch points in nodose neurons, interfering with NFκB signalling in SCG neurons affected neurite extension to a greater extent but had no effect on branching. This difference may be due to the manner of NFκB activation in the different neuronal types. While NFκB activation in nodose neurons is independent of BDNF, NGF has previously been shown to activate NFκB to promote neuronal survival (Maggirwar et al., 1998). It is likely that this activation route also occurs in the cultures described in this chapter as a comparable role for NFκB in the survival of SCG neurons is observed. Thus NGF-activated NFκB could be mediating some neurite outgrowth in SCG neurons.

In PC12 cells, more than one pathway leading from NGF exposure to NFκB activation has been described. Mamidipudi et al. describe the formation of a complex including both p75 and TrkA linked through IRAK, p62 and traf6, leading to activation of an atypical PKC, which phosphorylates IKKβ to cause NFκB release through IκB degradation (Mamidipudi et al., 2002; Mamidipudi and Wooten, 2002). An atypical pathway for the activation of NFκB by NGF has also been described (Bui et al., 2001). Rather than serine phosphorylation causing targeted degradation of IκBα, they showed that NGF-induced tyrosine phosphorylation of IκBα led to activation of NFκB without significant IκBα degradation. It is not known if NFκB is constitutively active in SCG neurons, as is the case nodose neurons. In future experiments it would be interesting to investigate whether NFκB is constitutively active in neonatal sympathetic neurons *in vitro*, and determine whether NGF causes additional activation of NFκB in these neurons. Also, it will be important to ascertain whether constitutive and induced NFκB activation in these neurons occurs via tyrosine or serine phosphorylation of IκBα.

The roles of NFκB in development are different in different neuronal populations. In nodose neurons NFκB is involved exclusively in neurite outgrowth, while in neurons of the SCG NFκB is involved in both survival and outgrowth. Additionally, the change in outgrowth induced by interfering with NFκB-activated gene transcription

in the two different populations appears different in character. For neurite outgrowth, however, the role of NFκB is restricted to a similar time window in both populations. In chapter 2, I showed that NFκB is only involved in promoting neurite outgrowth between E18 and P1, while in SCG neurons the effect of NFκB is also developmentally restricted, with no effect being detected at E17, but with a small effect still being detectable by P5 (D Gallager; personal communication). Although further clarification of this time window is necessary, a slightly later time window is consistent with NFκB being important for neurite outgrowth at the same stage of development of SCG neurons as occurs in nodose neurons, as neurons of the SCG develop slightly later than those of the nodose ganglia.

An interesting additional observation made from my studies of sympathetic neurons was that the broad-spectrum caspase inhibitor induced a decrease in total neurite length of between 19% and 28%, and a decrease in neurite branching of just under 40% in SCG neurons grown with NGF. While caspases play a key role in bringing about apoptosis, they have recently been implicated in neuronal growth and plasticity, as well as differentiation and cell cycle progression in a number of cell types (Fadeel et al., 2000; Fernando et al., 2002; Gilman and Mattson, 2002; Gulyaeva, 2004; Los et al., 2001). Specific caspases are localised in developing neurites and adult synapses (Shimohama et al., 2001a; Shimohama et al., 2001b; Yan et al., 2001) and caspase-3 has been found to be necessary for normal long-term potentiation in hippocampal neurons (Gulyaeva et al., 2003). Caspases have also been found to be important in long-term, but not short-term, spatial memory (Dash et al., 2000). Gilman and Mattson (2002) found that the use of a broad-spectrum caspase inhibitor in embryonic hippocampal neurons resulted in an increase in the rate of neurite extension and suggest that this is a consequence of caspase cleavage of proteins involved in neurite outgrowth including actin and members of the Ras and Rho families of GTPases. Therefore, while it is possible that the decrease in neurite outgrowth seen in our experiments could be due to a sub-toxic, but nevertheless deleterious, effects of the caspase inhibitor on neuronal health, it could also be that this effect reflects an involvement of caspases in neurite outgrowth. In other studies of SCG neurons, caspase inhibitors have been used to maintain neurons

during NGF deprivation without any observable changes in process morphology, although small reversible changes in the electrical properties of these neurons were detected (Werth et al., 2000). These studies, using different caspase inhibitors, have suggested that the involvement of caspases in neurite outgrowth vary. It will be interesting in future to see whether caspases are physiological regulators of neurite growth.

In summary, the data reported in this chapter shows that NFκB is involved in the regulation of neurite outgrowth, but not survival, in nodose neurons, while in sympathetic neurons, inhibition of NFκB signalling results in both decreased survival and neurite outgrowth. These findings indicate that NFκB may regulate one or more different aspects of neuronal development in different populations of neurons.

Chapter 4

Reg-2 is involved in the regulation of neuronal survival in the developing mouse peripheral nervous system.

4.1. Introduction

Reg-2 is a 65kDa protein, also called Reg-IIIβ in mice, or pancreatitis associated protein (PAP), peptide-23 or HIP (gene induced in hepatocellular carcinoma, intestine and pancreas) in rats and humans. It is expressed in developing sensory and motor neurons and its expression in the rat small intestine is developmentally regulated, corresponding principally with the period of high cell stress during weaning (Chakraborty et al., 1995; Livesey et al., 1997). Adult mice also express Reg-2 at lower levels in the small intestine, pancreas and colon (Narushima et al., 1997). In many cells its expression can be regulated by cytokines, particularly TNF-α, IL-6 and CNTF via MEK1 or STAT3 (TNF-α and IL-6/CNTF respectively (Chakraborty et al., 1995; Dusetti et al., 1995; Nishimune et al., 2000). Reg-2 has several physiological effects, with its predominant role being the promotion of growth and survival in a range of cell types. Reg-2 is anti-apoptotic for gastrointestinal epithelial and pancreatic acinar cells and in the nervous system it acts on both neuronal and non-neuronal cells, promoting Schwann cell mitogenesis and motor neuron survival (Christa et al., 1996; Livesey et al., 1997; Nishimune et al., 2000; Ortiz et al., 1998). Indeed, in cultured motor neurons Reg-2 has been found to be an obligatory signalling intermediate for CNTF-induced neuronal survival (Nishimune et al., 2000). As yet, no receptor for Reg-2 has been discovered. Reg-2 is detected in sera from patients suffering from hepatocellular carcinoma and is secreted by proliferating ductules, rat uterine and pituitary cells and during pancreatitis (Chakraborty et al., 1995; Christa et al., 1999; Iovanna et al., 1994; Katsumata et al., 1995). In the nervous system, application of the exogenous protein to cultured cells elicits survival effects, and *in vivo* anti-Reg-2 antibodies block regenerative effects after nerve injury (Livesey et al., 1997; Nishimune et al., 2000).

In both the developing and adult nervous system, neuropoietic cytokines have several roles, including proliferation, survival, differentiation, regeneration, maintenance of neurotransmitter phenotype and plasticity (reviewed in (English, 2003; Profyris et al., 2004; Stankoff et al., 2002; Turnley and Bartlett, 2000). In chapter 3 I demonstrated that NF-κB regulates the growth, but not survival, of newborn nodose neurons in response to CNTF. This suggests that at some point following the activation of the CNTF receptor complex, the pathways transducing survival- or neurite outgrowth-promoting effects in these neurons diverge. Current knowledge of CNTF-induced signalling pathways in other neuronal populations suggests that CNTF binding to the gp130-LIFRβ-CNTFRα complex induces janus kinases (JAK) to phosphorylate several tyrosine residues on the cytoplasmic tail of the gp130 protein. This creates docking sites for a range of proteins containing SH2 domains and subsequent activation of kinases such as PI3- kinase and MAP kinases, and transcription factors such as STAT-3 (Heinrich et al., 2003; Turnley and Bartlett, 2000). One point of divergence in the signalling of survival and outgrowth is potentially Reg-2. In motor neurons, exposure to CNTF leads to STAT-3 activation and Reg-2 production, with Reg-2 then promoting neuronal survival via PI3K and NFκB (Nishimune et al., 2000). In this population, Reg-2 expression occurs at a late stage in development, after target innervation is established, thus excluding Reg-2 from a major role in neurite outgrowth (Nishimune et al., 2000). Activation of other pathways, such as the MAP kinase cascade, could support neurite outgrowth. The results presented in chapter 3 already suggest that there are differences in the outgrowth- and survival-promoting signalling pathways induced by CNTF between central motor neurons and peripheral sensory neurons, as super-repressor IκBα does not affect sensory neuron survival. I therefore chose to investigate the relationship between Reg-2 and CNTF in nodose neurons and trigeminal neurons, which also respond to CNTF.

4.2. Materials and Methods

4.2.1. Neuronal culture

Dissociated cultures of nodose and trigeminal ganglia neurons were set up from embryos and early postnatal mice obtained from overnight matings of CD1 mice, and cultured in the manner described in chapter 2. All the cultures were established in 35mm culture dishes except for those used in the initial dose-response experiment. This experiment was performed in 35mm dishes containing four 11mm wells in order to conserve Reg-2 protein, as limited quantities were available. This protein was a gift from C. Henderson (INSERM U382, IBDM, Marseille, France) and had been purified from adult rat pancreatic juice (Nishimune et al., 2000). The use of 4-well dishes is described for the SCG cultures in chapter 3.

The murine trigeminal ganglia are located in the base of the skull. The dissections were performed differently depending on the age of the embryo. Up to E14, the trigeminal ganglia were isolated by using tungsten needles (E12) or fine scissors (E14) by making two coronal incisions, one just above the eye, and the other between the maxillary and mandibular processes of the first brachial arch (figure 4.1). The trigeminal ganglia are thus revealed as two opaque structures in the this tissue slice which can be freed from the tissue slice and cleaned of any remaining connective tissue using tungsten needles. Ganglia from mice of E16 or over were dissected by using fine scissors to remove the top of the skull, after which the brain was removed. This revealed the trigeminal ganglia, resting in two niches in the base of the skull. These ganglia were removed using forceps and cleaned of all connective tissue using tungsten needles.

Dissected ganglia were trypsinized and triturated as described in chapter 2. Trypsinisation time varied from 8 to 30 minutes depending on the age of the mice. All trigeminal ganglia of E18 and over and nodose ganglia at P1 and older destined for RT-PCR were purified using differential sedimentation techniques. For this purification, trypsinized ganglia were washed in F12 plus 10% HIHS to inactivate the trypsin, then in CMF-HBSS and trituration performed in 1ml CMF-HBSS. This

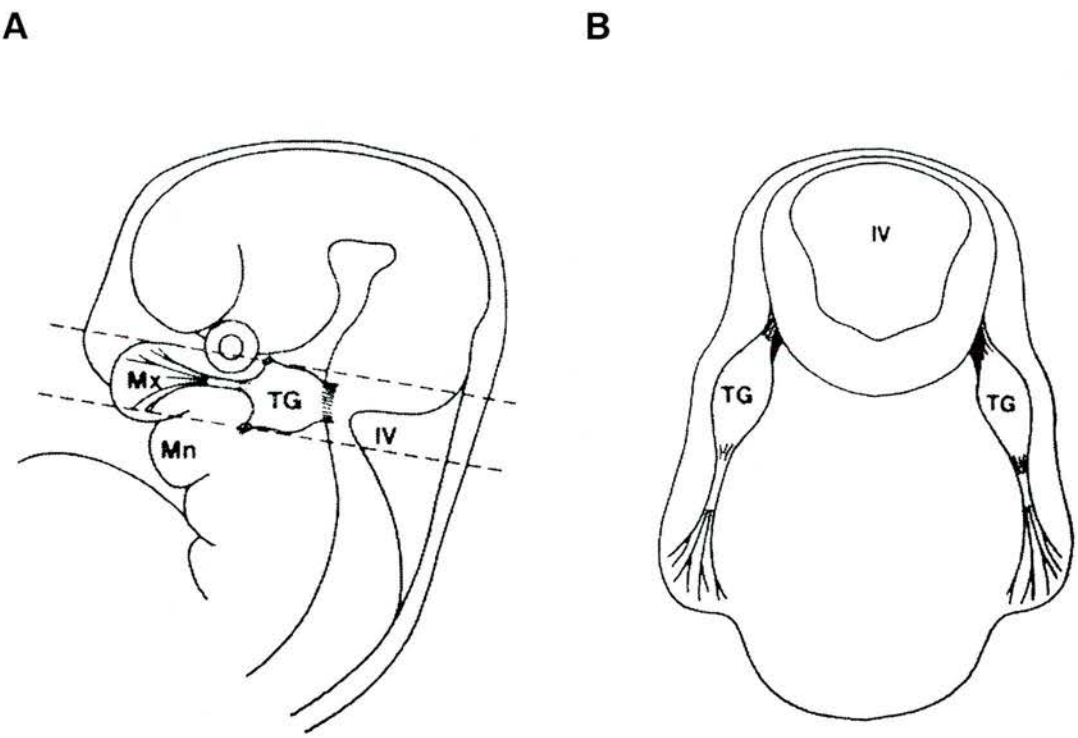

Figure 4.1 *Dissection of the trigeminal ganglia*
A) The lateral aspect of an E11 head showing the location of the transverse incisions made in order to obtain a tissue slice containing the trigeminal ganglia. B) The rostral aspect of the tissue slice containg the trigeminal ganglia (TG). Mx, Maxillary process; Mn Mandibular process; IV fourth ventricle. Taken from Davies, 1995

cell suspension was layered on top of 50-60ml F14 + 10% HIHS which had been placed in a 100ml glass dropping funnel on a vibration free platform at 4°C the previous evening. After one hour, the medium was carefully run off the column in 5ml aliquots, and the fractions containing neurons or non-neuronal cells identified. The neuronal fractions were then pooled and the cells plated or immediately collected for RNA extraction as required.

4.2.2. Genotyping transgenic mice

IKK-α and p65 knockout embryos were generated by mating mice heterozygous for null mutations in the relevant gene. Deletion of STAT-3 causes embryonic lethality at E6.5 (Takeda et al., 1997), therefore to study the effects of STAT-3 deletion at a later age I used a strain of STAT-3 conditional mutant mice used by (Alonzi et al., 2001) where STAT-3 expression was eliminated after gastrulation, but before E12.5 by the use of balancer strain of cre-transgenic mice. Mice were generated by matings crossing $bal1^{+}/STAT3^{wt/fl}$ males with $bal1^{-}/STAT3^{fl/fl}$ females. Embryos inheriting the $bal1^{+}$ and $STAT3^{fl}$ allele, which would have been deleted in the male, in combination with $STAT3^{fl}$ allele from the female, showed a significant reduction in STAT-3 expression. Mice of the $Bal1^{-}$, $STAT3^{wt/fl}$ genotype were used as a positive control. Pregnant females were killed by carbon dioxide asphyxiation, followed by cervical dislocation, after 18 days of gestation for IKK-α and STAT-3 mice, or 14 days gestation for p65 mice. Tissue samples were collected for each embryo and DNA extraction performed using the Nucleospin Tissue DNA extraction kit (Macherey-Nagel, Germany) following the manufacturers instructions. Briefly, cell were lysed by incubation of the tissue sample overnight in an SDS / proteinase K. DNA was then extracted by incubating the lysate with a silica membrane supplied as part of a spin column, and contaminants removed by washing with an ethanol-based solution. Finally DNA was eluted from the column using the low-ionic strength buffer supplied.

PCR was again used to identify the genotype of each embryo. The IKK-α genotyping was performed as described in chapter 2. Genotyping of the p65 knockout mice was performed in a similar manner, with altered annealing temperatures and cycle

numbers (see appendix). This genotyping used 3 primers (5'-CCTATAGAGGAGCAGCGCGGG-3'; 5'-AAATGTGTCAGTTTCATAGCCTGAAGAACG-3'; 5'-AATCGGATGTGAGAGGACAGG-3') producing two distinct products, that were separated on a 3% agarose gel, a 390bp product corresponding to the wildtype genotype and a 550bp product corresponding to the knockout product. Genotyping the STAT-3 mice required three different reactions. Reaction one detected the bal status of the mice, reaction two detected the presence of wildtype or fl alleles, and reaction three detected the presence of the deleted allele

4.2.3. RNA isolation and purification

RNA isolation and purification was performed according to the extraction method described by (Chomczynski and Sacchi, 1987). Ganglia or cells were lysed in 500µl of solution D (4M guanidinium thiocyanate, 25mM tri-sodium citrate pH 7.0, 0.5% N-laurylsarcosine, 0.1M 2-mercaptoethanol). Ganglia from older ages were also passed through a 25-gauge needle fitted with a 1ml syringe to ensure complete homogenisation. The following solution were added sequentially: 2µl of 5µg/ml *E. coli* tRNA; 50µl of 2M sodium acetate (pH4.4); 500µl of water saturated acidic phenol and 150µl of 25:1 chloroform:isoamyl alcohol. This mixture was shaken vigorously and incubated on ice for 5 minutes, then centrifuged at 15,000 rpm for 10 minutes. The upper aqueous phase, containing the total RNA, was transferred to a fresh microfuge tube and the RNA precipitated by the addition of two volumes of ethanol and incubated at -20°C overnight. The ethanol mixture was centrifuged at 15,000rpm for 30 minutes, to recover the total RNA from the mixture. The supernatant was discarded and the RNA pellet washed in 70% ethanol and air-dried. In order to remove all contamination with genomic DNA, the pellet was resuspended in a 50µl volume of 40mM Tris pH7.5, 6mM $MgCl_2$ 20mM vanadyl-ribonucleoside complex (VRC, an RNase inhibitor) and 37.5 units of RNase-free DNase I (Pharmacia) and incubated at 37°C for 90-120 minutes. The RNA was then purified using the RNaid kit (Bio101), as per manufacturers instructions. Briefly, 3 volumes of 3M $NaClO_3$ were added to the RNA solution followed by 30µl of RNA binding matrix for whole ganglia, or 20µl for cultured cells. This was mixed thoroughly and

incubated at room temperature for 15 minutes to allow the RNA to bind to the matrix. The matrix was sedimented by centrifugation and washed twice using the supplied washing solution. The solution was discarded and the RNA eluted from the matrix by the addition of 30µl of DEPC-treated water for cultured cells, or 100µl for whole ganglia and incubation at 60°C for 2-3 minutes. Following centrifugation, the supernatant, containing the total RNA, was transferred to a fresh tube and stored at -80°C until required.

4.2.4. Quantification of Reg-2 mRNA levels using competitive RT-PCR

RT-PCR is a sensitive technique for the detection of rare mRNA species in a total RNA extract from a small amount of tissue. The technique used for this study uses a cRNA internal control (competitor) to control for variations in amplification from reaction to reaction. The competitor is synthesised from the mRNA of interest, so that the sequence of the competitor differs by only 3-4% in the area to be amplified in the PCR reaction. This ensures that the kinetics of the amplification reactions for the two species are as similar as possible (previously described in (Wyatt et al., 1997; Wyatt and Davies, 1993). The reverse transcription mix is spiked with known quantities of the cRNA competitors for the gene of interest and a constitutively expressed housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) before reverse transcription is carried out. Two separate PCR reactions are then performed, one for the gene of interest, and the other for GAPDH. The products of the RT-PCR reactions are separated using acrylamide gel electrophoresis and the quantity of the natural species determined as a ratio of that of the competitor species. Before the final reaction is performed, the amount of the competitor species to be added must be titrated against that of the natural species, as the response of the imaging system is only linear over a small range. The aim is to have no more than a 5x difference between the intensity of the two amplified products. The amount of product for the mRNA of interest is expressed relative to the levels of GAPDH in the sample, thus controlling for different amounts of total RNA present in the original samples.

4.2.4.1. Competitor synthesis

The mouse Reg-2 mRNA was cloned into pBluescript SK+ and kindly donated by the lab of C. Henderson (INSERM U.382, Marseilles). To obtain a sequence 3-4% longer than the natural species for the RT-PCR assay 3-4 base pairs had to be added to the middle of the cloned sequence. This was done by digesting the plasmid with NcoI (Promega), a restriction enzyme with a single site of restriction located 237bp into the 486bp insert, to produce a 4bp 5' overhang. The resultant DNA was purified using the MIDAS CleanIt kit (Biogene), the overhang was the filled in using Klenow polymerase, repurified and the blunt ends religated using reagents from the pGEM-T vector kit (Promega). The resulting plasmids were then used to transform JM109 competent cells (Promega). First, however, a digestion was again performed using NcoI, this linearised unfilled plasmids, but did not affect filled plasmids, thus decreasing the chances of producing transformed colonies containing the unfilled plasmid. After overnight incubation at 37°C, single ampicillin-resistant colonies were picked and used to inoculate 5ml of LB broth containing ampicillin (50μg/ml). These were incubated overnight at 37°C and the plasmid DNA extracted using the MIDAS Plasmidspin kit (Biogene) as per manufacturers instructions. To verify that the plasmid contained the fill-in, a digestion was again performed using NcoI and the digest analysed using gel electrophoresis. The plasmid was then sent for sequencing (MWG-Biotech) to determine the size of the insert, which was 4bp for the Reg-2 competitor.

The last step of competitor synthesis was to perform an *in vitro* transcription to synthesise the cRNA. To produce run-off transcripts of a defined length, the plasmid was linearised at the 3' end of the inserted DNA using EcoRI (Promega), the digest analysed using gel electrophoresis and the plasmid band cut out of the gel and purified using the MIDAS CleanIt kit (Biogene). *In vitro* transcription was performed using T7 RNA polymerase (Promega), in a reaction mix also containing RNAguard (Pharmacia). The *in vitro* transcription was carried out for 2 hours at 37°C, after which VRC and DNaseI were added and a further 2 hour incubation at 37°C was performed in order to degrade the plasmid DNA. The RNA transcripts were purified using the RNaid kit (Bio101) as described earlier, and the purified

RNA recovered in 100µl of DEPC-treated water. 10µl of the transcript were run on a 1% RNase free agarose gel to check the integrity of the transcript and a further 10µl were used to determine the RNA concentration spectrophotometrically. The remaining RNA was diluted with water and precipitated at a concentration of 1ng/µl by the addition of an appropriate volume of water, 0.1 volumes of 3M sodium acetate (pH 5.5), 3 volumes of ethanol and 20ng E. coli tRNA for every 1ng of transcript. The ethanol-precipitated transcripts were then kept at -20°C until use. The GAPDH competitor had been synthesised previously and is described in (Wyatt et al., 1997).

4.2.4.2. The competitive RT/PCR assay

The first part of the RT/PCR assay is to reverse transcribe total RNA from the experimental samples in combination with the cRNA for the genes of interest. A reaction mix was prepared containing total RNA (between 1 and 10µl depending on the concentration of the sample), a known amount of cRNA competitor, 500µM dNTPs (MBI Fermentas), 10µM random hexanucleotides (Pharmacia), 10mM DTT and 5xBuffer (supplied with the superscript reverse transcriptase, Gibco BRL) and made up to a total volume of 40µl per reaction. The reverse transcription was performed by incubating the reaction mix for 2 minutes at 90°C, in order to break down any secondary structure within the RNA, before the addition of 1µl of Superscript reverse transcriptase per tube, except for those tubes that were to serve as negative controls. These tubes contained everything except the enzyme, in order to check for the presence of contaminating genomic DNA, which would be amplified in the PCR reaction even in the absence of reverse transcriptase. Addition of the enzyme was immediately followed by a 90 minute incubation at 37°C, after which the reaction was halted by denaturation of the enzyme by a 6 minute incubation at 95°C.

The second part of the RT-PCR assay was to amplify the cDNAs for Reg-2 and GAPDH, using a separate PCR reaction for each product. Two primers were used for each reaction, designed to span the region of the insert in the competitor. Each reaction therefore produced two different species, the competitor producing a species that was 4bp longer than the natural product. The products of the Reg-2 reaction

were 84 and 88 bp in length, whereas those of the GAPDH reaction were 97 and 101 bp long. The GAPDH primers and reaction conditions have been described previously (Wyatt and Davies, 1993). The details of the Reg-2 reaction can be found in appendix 1. After amplification the two species in each reaction were separated by electrophoresis on an 8% non-denaturing polyacrylamide gel and visualised, after a 15 minute incubation with the non-isotopic UV-sensitive dye SyberGold. UV illumination and a computer-controlled video-imaging system were used in conjunction with the Phoretix 1D quantifier software to assess individual band intensity.

4.2.5. mRNA expression studies using semi-quantitative RT-PCR

Semi-quantitative RT-PCR was used to investigate the expression of other Reg family genes in the nodose and trigeminal ganglia. Unlike competitive RT-PCR, this does not use an external competitor to determine the absolute concentration of mRNA in a sample, rather it expresses the levels of the mRNA of interest in terms of the levels of expression of a control mRNA, in this case GAPDH, whose expression has been found to be constant. The amplification of both products is carried out in the same reaction mix, thus controlling for both different levels of DNA in differing samples and the different efficiency of amplication between separate reactions. The products amplified were designed to be approximately 100bp different in size so that they could be distinguished by agarose gel electrophoresis. Agarose gels were stained using ethidium bromide, imaged using a computer controlled UV imaging system and quantified using the Phoretix 1D Quantifier software. The reverse transcription and PCR reactions were performed using the same basic protocols described earlier and details of reaction mixtures and cycling times can be found in the appendix.

4.2.6. Western blotting

Polyacrylamide gel electrophoresis (PAGE) followed by immunolabelling was used to analyze the phosphorylation, and therefore activation state, of Akt and Erk1/2 in cultured neurons stimulated with Reg-2 for 5, 15 and 30 minutes.

P1 nodose neurons and purified P1 trigeminal neurons were established in dissociated culture on a polyornithine/laminin substratum on 24-well plates in 1ml defined medium. The neurons were cultured in the absence of trophic support for 3 hours to permit attachment, then stimulated for 5, 15 or 30 minutes with 1 ng/ml Reg-2, before finally being washed in PBS and lysed in 25µl lysis buffer. Each sample was collected in lysis buffer and prepared for electrophoresis by sonication for 1 minute followed by the addition of loading buffer. The samples, as well as a protein standard, were then loaded onto a pre-prepared 8 by 6cm running gel (8%) with a 3% loading gel above and electrophoresis performed at 25mA until the bromophenol blue dye front in the loading buffer reached the bottom of the gel. Protein was then transferred to a polyvinylidene difluoride (PVDF) membrane (BDH Poole, UK) using electrophoretic elution and any non-specific binding sites blocked by a 1 hour incubation at room temperature in 0.01% tween-tris buffered saline (T-TBS) containing 5% non-fat dried milk powder. The membrane was washed three times in T-TBS and incubated over two nights with a 1:1000 dilution of rabbit anti-phospho-Akt antibody (Cell Signalling Technology, UK) in T-TBS. After another set of washes the membrane was then incubated with a 1:5000 dilution of a horseradish-peroxidase-linked donkey anti-rabbit IgG secondary antibody (Amersham Biosciences, UK) diluted in T-TBS containing 5% non-fat dried milk powder for three hours. Binding was then detected using the ECL+ kit (Amersham Pharmacia Biotech, UK) as per manufacturers instructions and an autoradiography film exposed to the membrane for between 5 and 15 minutes, before the film was developed. The membrane was then stripped by incubating the membrane in 100mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl (ph6.7) for 30 minutes at 70°C before reblocking, reprobing with first rabbit anti-phosho-Erk1/2, followed by a rabbit pan-Akt antibody (Cell Signalling Technology, UK) as a loading control.

4.2.7. Quantification of neurite outgrowth.

P1 trigeminal neurons were established in low density cultures and after 24, 48 and 72 hours the neurons were fixed and immunocytochemistry performed as described in chapter 2.2.6, to label the neurons for βIII-tubulin. The neurons were then imaged using the Zeiss 510 laser-scanning microscope and neurite outgrowth quantified

using the Sholl analysis, for the total neurite length and the number of branch points. In this study the rings used for the Sholl analysis are 50μm apart, rather than the 30μm used in the earlier chapters.

4.3. Results

4.3.1. Reg-2 expression is regulated by IL-6 type cytokines in peripheral sensory neurons

My investigation into the role of Reg-2 in the peripheral nervous system began by studying the expression of Reg-2 in two sensory neuron populations, those of the nodose and trigeminal ganglia. I also investigated the expression of Reg-2 in three signalling knockouts, p65, IKKα and STAT-3 and whether its expression was regulated by cytokines, as has been suggested for motoneurons (Nishimune et al., 2000). These studies used competitive RT-PCR to assess the levels of Reg-2 mRNA expressed in either whole ganglia or dissociated neuronal cultures.

4.3.1.1. Reg-2 mRNA is expressed in both the nodose and trigeminal ganglia during a restricted developmental period

The first step in this investigation was to look at the expression of Reg-2 mRNA in nodose neurons throughout development. Nodose and trigeminal ganglia were dissected from embryos between E12 and E18 and pups at P1 and P5, and the total RNA extracted. Competitive RT-PCR was then used to quantify the levels of mRNA for Reg-2 and the housekeeping gene, GAPDH. Figure 4.2 shows that Reg-2 mRNA was expressed in both nodose and trigeminal ganglia. In nodose ganglia Reg-2 mRNA was barely detectable at E12 and E14, after which its expression increased to a peak of 5 fg/pg GAPDH at E18 ($p<0.05$), with levels decreasing to barely above the baseline by P5. In trigeminal ganglia, reg-2 expression had increased significantly by E14 ($p<0.05$), although the level at this age was small compared to later ages. By E16 the level of reg-2 mRNA expression had increased to 18 fg/pg GAPDH, over 3 times the peak expression levels seen in nodose ganglia. Reg-2 mRNA expression in trigeminal ganglia then stayed at a plateau until P1 after which levels fell rapidly, so that by P5 the levels of expression were similar to those at E14. These results show that reg-2 mRNA is expressed in peripheral sensory neurons during a restricted period of development, from the late embryonic to early postnatal stages.

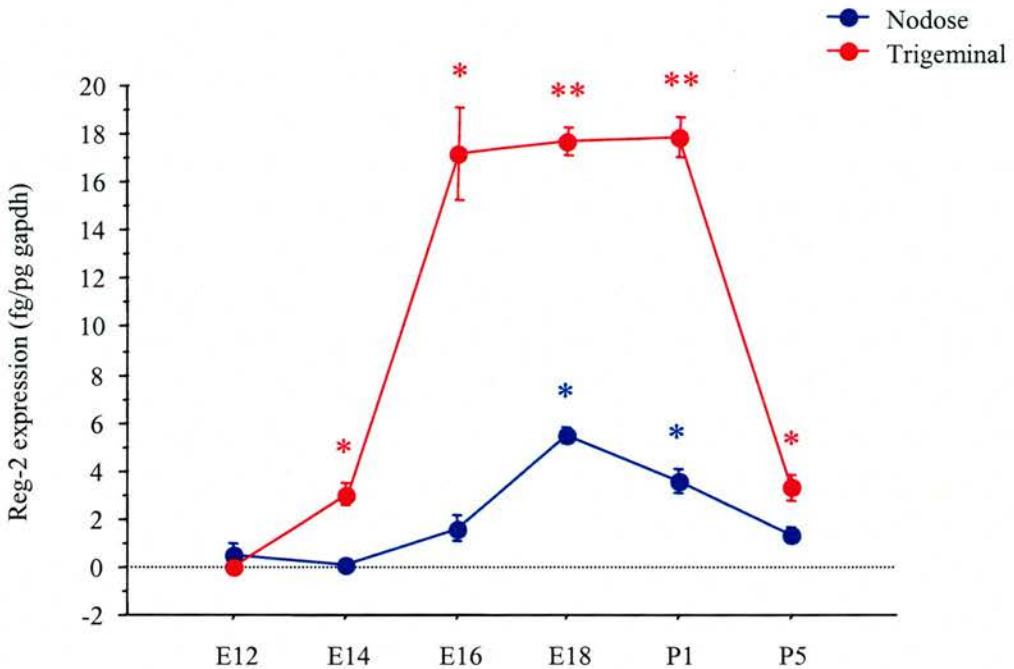

Figure 4.2 *Reg-2 mRNA is expressed in both the nodose and trigeminal ganglia during a restricted developmental period.*
Nodose and trigeminal ganglia were dissected from mice between E12 and P5 and their expression of Reg-2 mRNA assessed using competitive RT-PCR. The means and standard deviations of data obtained from 3-5 individuals at each age are shown. Statistical comparisons shown are with respect to E12 ganglia, * $p<0.05$, ** $p<0.001$

4.3.1.2. Reg-2 expression in sensory ganglia is dependent on STAT-3, but not IKK-α or p65 expression.

The next investigation examined whether several intracellular signalling proteins downstream of CNTF receptor activation are involved in the regulation of Reg-2 expression *in vivo*. The JAK/STAT and NFκB intracellular signalling pathways were studied by assessing the Reg-2 mRNA levels in ganglia isolated from transgenic embryos lacking STAT-3, IKK-α or p65. Figure 4.3A shows the levels of Reg-2 mRNA expression in E18 ganglia dissected from embryos from conditionally deleted STAT-3 mice possessing 2 (Bal-, wt/fl), 1 (Bal-, fl/Δ) or 0 (Bal+, fl/Δ) active copies of the STAT-3 gene. Here, the results are expressed as a percentage of the Bal-, wt/fl group for each ganglion, to allow comparison between ganglia. This was necessary because the absolute levels of Reg-2 mRNA were again substantially higher in the trigeminal than in the nodose ganglia. In trigeminal ganglia a 45% decrease in Reg-2 mRNA expression was found in embryos carrying only one active copy of the gene ($p<0.05$) and a 80% decrease was seen in mice carrying two inactivated STAT-3 genes ($p<0.001$). Similar mean decreases can be seen in nodose ganglia (30% and 75% respectively), however these decreases did not reach significance. This is most likely to be because the very low quantities of Reg-2 mRNA in these ganglia makes detection difficult and increases the effects of variation between samples. In neither ganglion was Reg-2 expression completely abolished in the STAT-3 conditional knockout mice. Conditional inactivation can, however, be incomplete, and the extent of the inactivation can vary from cell to cell, therefore some residual Reg-2 expression might be expected due to incomplete activation in some cells.

Nodose and trigeminal ganglia were also dissected from IKK-α mice at E18 and from p65 mice at E14, and the levels of Reg-2 mRNA expression quantified (figure 4.3B and 4.3C, respectively). No significant differences in Reg-2 mRNA levels could be detected between wildtype and knockout nodose and trigeminal ganglia from IKK-α embryos or p65 wildtype and knockout trigeminal ganglia. Reg-2 expression in nodose ganglia at E14 is virtually nonexistent, therefore the levels of Reg-2 mRNA expression could not be assessed in the p65 knockout mice and these mice die *in utero* after this age. These results suggest that *in vivo* Reg-2 expression in

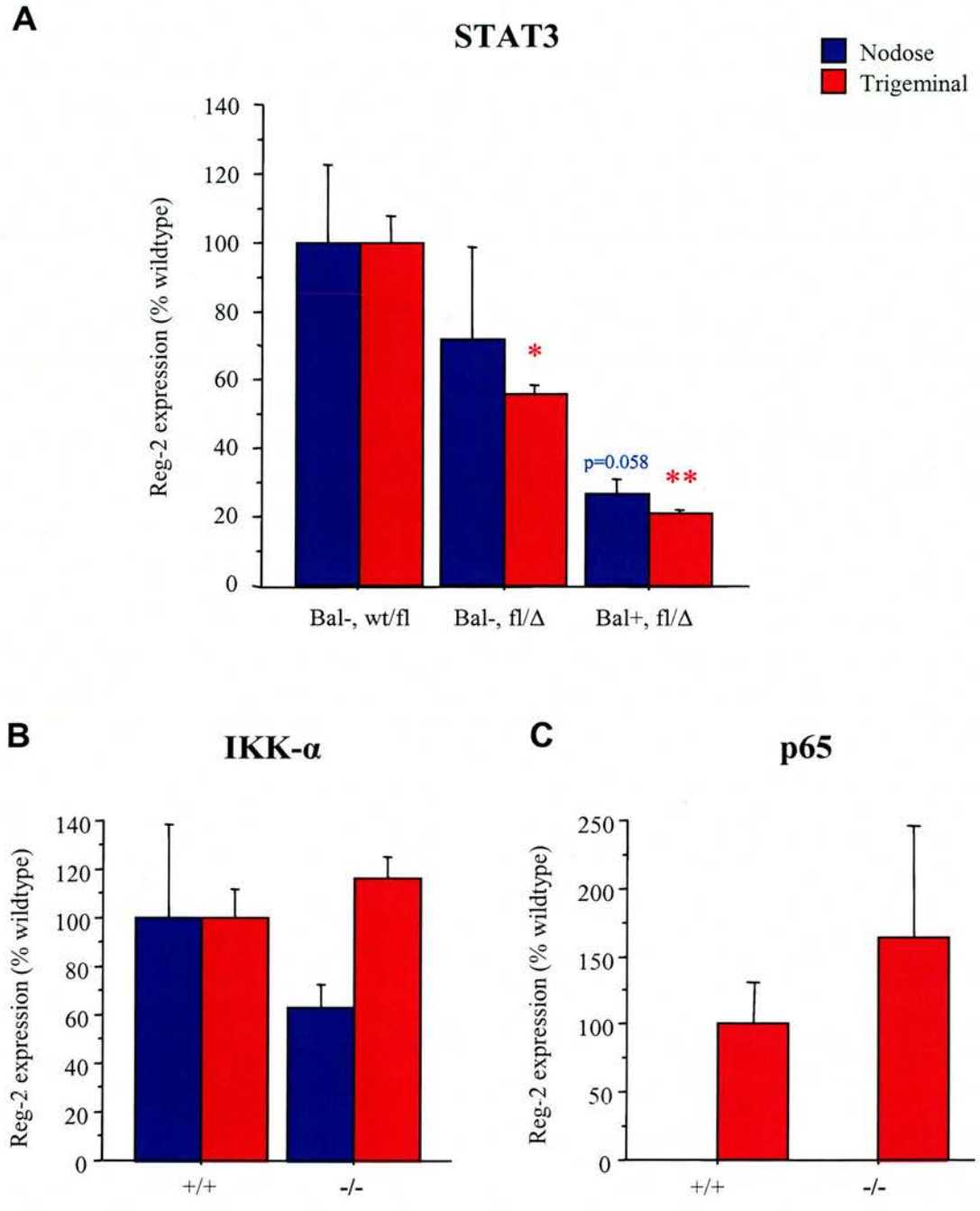

Figure 4.3 *Reg-2 expression in sensory ganglia is dependent on STAT-3, but not IKK-α or p65, expression.*

Nodose and trigeminal ganglia were dissected from STAT-3, IKK-α or p65 knockout mice at either E18 (STAT-3, IKK-α) or E14 (p65) and Reg-2 mRNA expression assessed using competitive RT-PCR. A) Reg-2 expression in STAT-3 conditionally deleted mice. B) Reg-2 expression in IKK-α knockout mice. C) Reg-2 expression in p65 knockout mice. The levels of Reg-2 expressed are shown relative to the wildtype mice for each ganglion to allow for comparison and the means and standard deviations of data obtained from 3-6 individuals per group shown. Statistical comparisons shown are with respect to wild-type ganglia, * $p<0.05$, ** $p<0.001$

peripheral sensory neurons is regulated via the JAK/STAT, but not the NFκB signalling pathway.

4.3.1.3. Reg-2 expression in sensory neurons is maintained by cytokine, not neurotrophin stimulation

To investigate whether CNTF was capable of directly regulating neuronal Reg-2 expression *in vitro,* purified sensory neurons were cultured in the presence of combinations of neurotrophins and cytokines and their expression of Reg-2 mRNA quantified. Purified P1 nodose and trigeminal neurons were cultured for 3 hours in the absence of trophic factors, an initial sample of neurons collected and the remaining culture dishes supplemented with CNTF or LIF (50ng/ml), BDNF or NGF (10 ng/ml) respectively, or a combination of a cytokine and the relevant neurotrophin. After 24 hours in culture the neurons were lysed, the lysate collected, and the levels of Reg-2 mRNA quantified and compared to the level of Reg-2 mRNA expression in the initial samples. In Figure 4.4A the levels of reg-2 mRNA in nodose and trigeminal neurons cultured with combinations of CNTF and the relevant neurotrophin are shown. The levels of reg-2mRNA levels are expressed as a percentage of the mean level of expression after 3 hours in culture. This is to allow comparison between nodose and trigeminal neurons because the absolute levels of expression in the trigeminal neurons after 3 hours were 7 times greater than in the nodose neurons. In both the nodose and trigeminal neurons cultured for 24 hours in the presence of CNTF, the same level of reg-2 mRNA expression was detected as at the initial time point. In contrast, when these neurons were cultured in the presence of neurotrophins, decreased levels of Reg-2 expression were seen. In nodose neurons there was a 50% decrease in Reg-2 expression ($p<0.05$) and in trigeminal neurons this decrease was 55% ($p<0.001$). Interestingly, when each type of neuron was subject to a 24 hour incubation in the presence of both CNTF and either BDNF (nodose) or NGF (trigeminal), there was a difference in the response seen in the two neuronal populations. For nodose neurons cultured in the presence of CNTF and BDNF the level of reg-2 expression seen after 24 hours was decreased to 50% of that seen in the initial sample ($p<0.05$), similar to the level seen in neurons cultured in media supplemented with BDNF alone. In contrast, trigeminal neurons cultured for

24 hours in the presence of CNTF and NGF showed levels of Reg-2 expression that were not significantly different from those of the initial sample, or of neurons cultured with CNTF alone. The neuronal survival in these cultures was also monitored and there were no significant differences in survival between any of these conditions in either ganglion (Fig 4.4 C).

The same set of experiments was repeated replacing CNTF with LIF, to see if a similar expression pattern could be detected with another member of the cytokine family. These results are seen in figure 4.4B. In nodose neurons there was a tendency towards decreased Reg-2 expression in neurons cultured in the presence of LIF alone, as compared to the initial time point (40%), although this decrease is not significant. Significant decreases in Reg-2 mRNA expression, however, were seen in nodose neurons incubated in defined medium supplemented with BDNF or BDNF plus LIF (70 and 60% decrease respectively, $p<0.05$). Trigeminal neurons showed a tendency towards increased Reg-2 mRNA expression when cultured in the presence of either LIF or LIF + NGF (20 and 15% respectively), although these increases were not significant. When cultured in the presence of NGF alone trigeminal neurons again showed a 52% decrease in Reg-2 mRNA expression ($p<0.05$). Again there were no significant differences in the survival of neurons of different groups from either ganglion (fig 4.4D). Broadly speaking, these results show the same trends as those seen in neurons cultured with combinations of CNTF and neurotrophins. Together the results from the CNTF and LIF cultures suggest that Reg-2 expression is maintained in nodose and trigeminal neurons cultured in the presence of cytokines,

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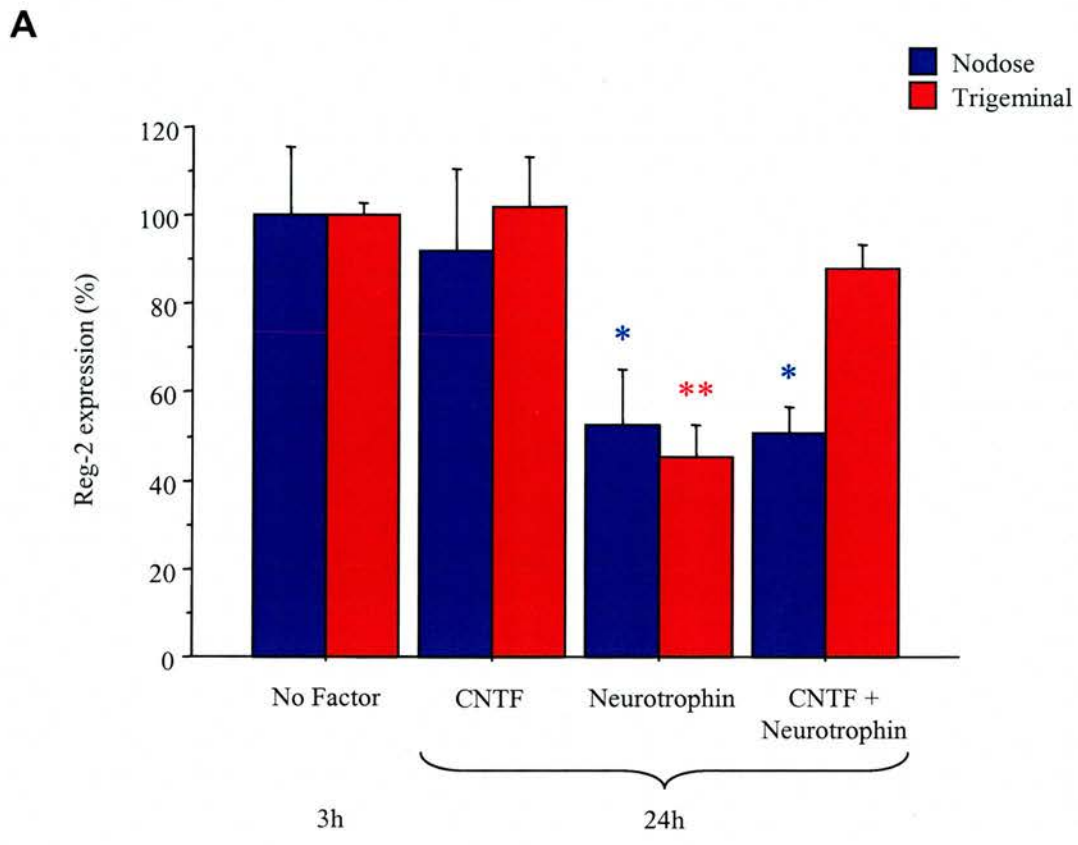
* **Figure 4.4** *Reg-2 expression in sensory ganglia is maintained by cytokine, but not neurotrophin stimulation.*
Purified P1 nodose and trigeminal ganglia were cultured in the presence of cytokines or neurotrophins, or both, for 24 hours and their level of Reg-2 mRNA expression quantified using competitive RT-PCR. A) Reg-2 expression in neurons cultured with CNTF (50 ng/ml), BDNF or NGF (10 ng/ml, nodose and trigeminal ganglia respectively) or both. B) Reg-2 expression in neurons cultured with LIF (50 ng/ml), BDNF or NGF (10 ng/ml, nodose and trigeminal ganglia respectively) or both. C) and D) Percent survival after 24h in culture for the cultures in (A) and (B). The levels of Reg-2 are expressed relative to the 0h control group to allow for comparison and the means and standard deviations of data obtained from 3 duplicate cultures are shown. Statistical comparisons shown are with respect to the 0h group, * $p<0.05$, ** $p<0.001$


A
Nodose
Trigeminal
Reg-2 expression (%)
120
100
80
60
40
20
0
*
**
*
No Factor
CNTF
Neurotrophin
CNTF +
Neurotrophin
3h
24h

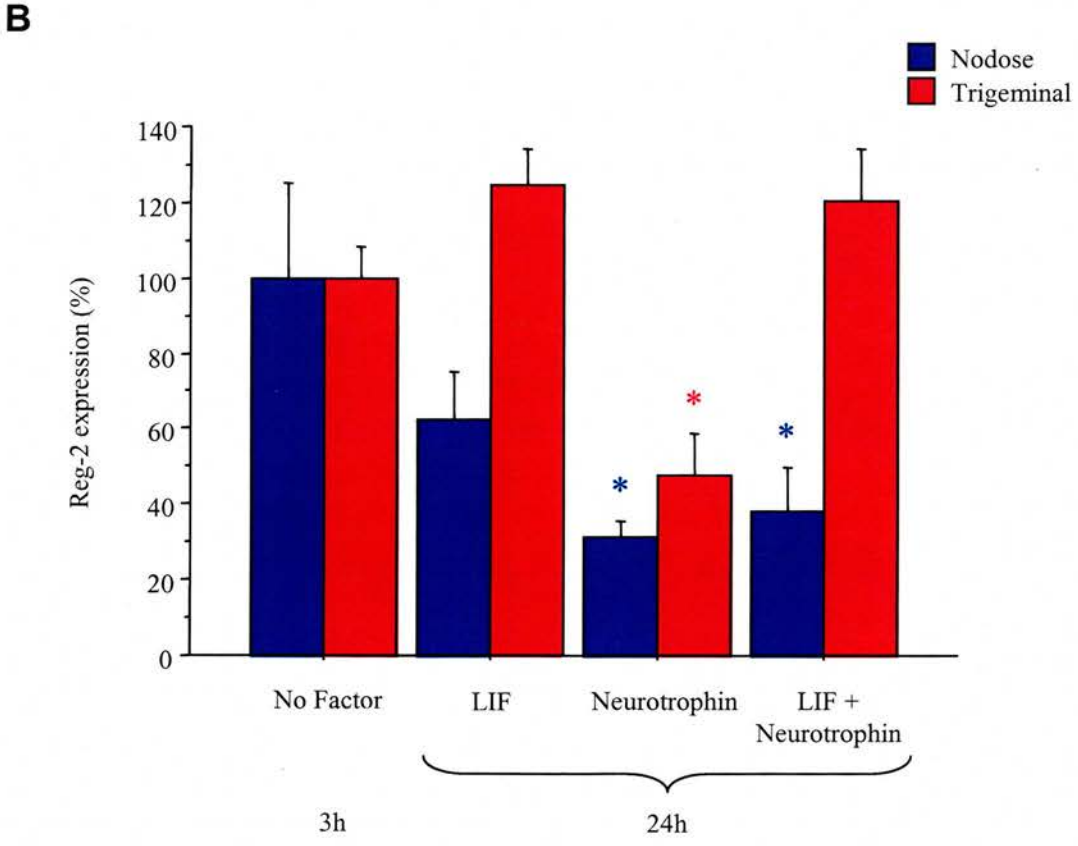
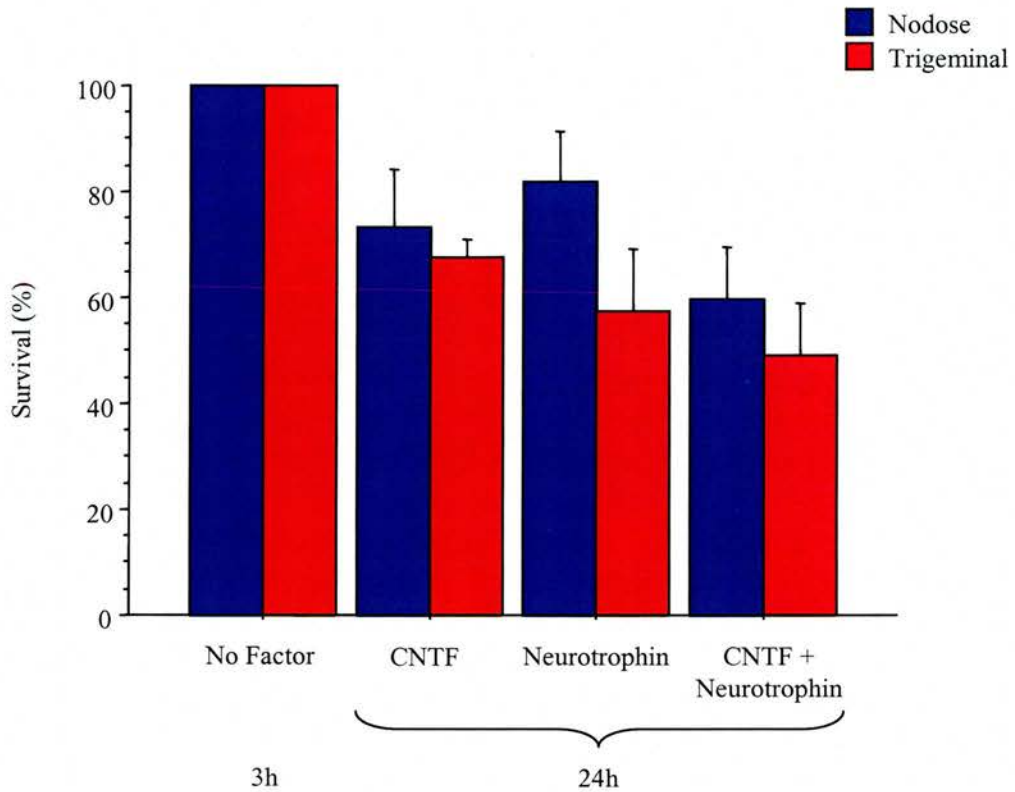

B
Nodose
Trigeminal
Reg-2 expression (%)
140
120
100
80
60
40
20
0
*
*
*
No Factor
LIF
Neurotrophin
LIF +
Neurotrophin
3h
24h

Figure 4.4

C


Nodose
Trigeminal
Survival (%)
100
80
60
40
20
0
No Factor
CNTF
Neurotrophin
CNTF +
Neurotrophin
3h
24h

D

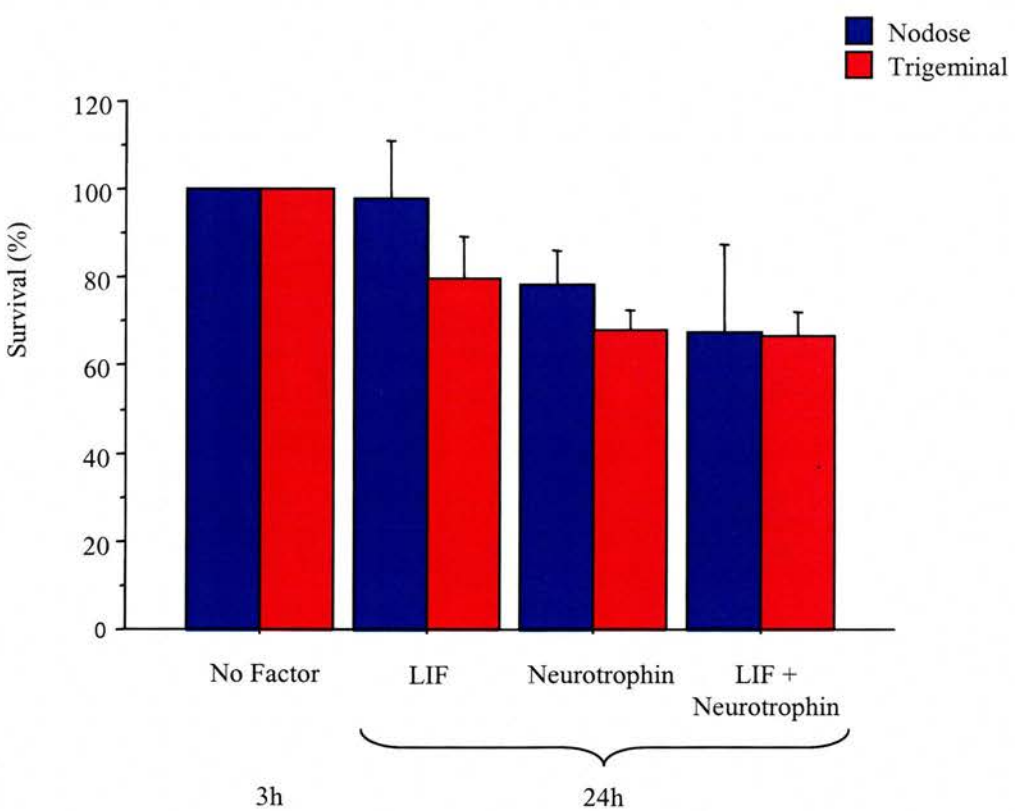

Nodose
Trigeminal
Survival (%)
120
100
80
60
40
20
0
No Factor
LIF
Neurotrophin
LIF +
Neurotrophin
3h
24h

Figure 4.4

but decreased in neurons cultured with neurotrophins. The results seen when the two populations are cultured with both types of factors together suggest that the signalling events in each type of neuron vary. In trigeminal neurons the results suggest that cytokines actively promote the maintenance of reg-2 expression, and that neurotrophins are not involved in reg-2 expression, whereas those in the nodose neurons might suggest that BDNF is actively suppressing reg-2 expression, which may be maintained either by default or through CNTF-induced gene expression.

To further investigate the above question, cultures of purified P1 nodose and trigeminal neurons were established in the same manner. However in these experiments, all dishes were incubated for 24 hours in the presence of 10 ng/ml BDNF or NGF respectively. After 24 hours, the neurons were washed twice in unsupplemented media and cultured for a further 24 hours with either CNTF, neurotrophin or both. Reg-2 expression was quantified in neurons after 3 hours in defined media without additional trophic support, at 24 hours, and at 48 hours. The results of this experiment can be seen in figure 4.5. After 24 hours in culture with BDNF or NGF, the expression of Reg-2 had decreased to less than 20% of the level of expression at the initial time point. After a further 24 hours in culture with CNTF, neurotrophin or both there were no further changes from this low level of expression in any group. These results, when compared to the earlier 24h results, are ambiguous and further experiments, such as starting with a 24 hour incubation with CNTF then switching to neurotrophin would be needed before the roles of neurotrophins and cytokines in regulating reg-2 expression in vitro can be further defined.

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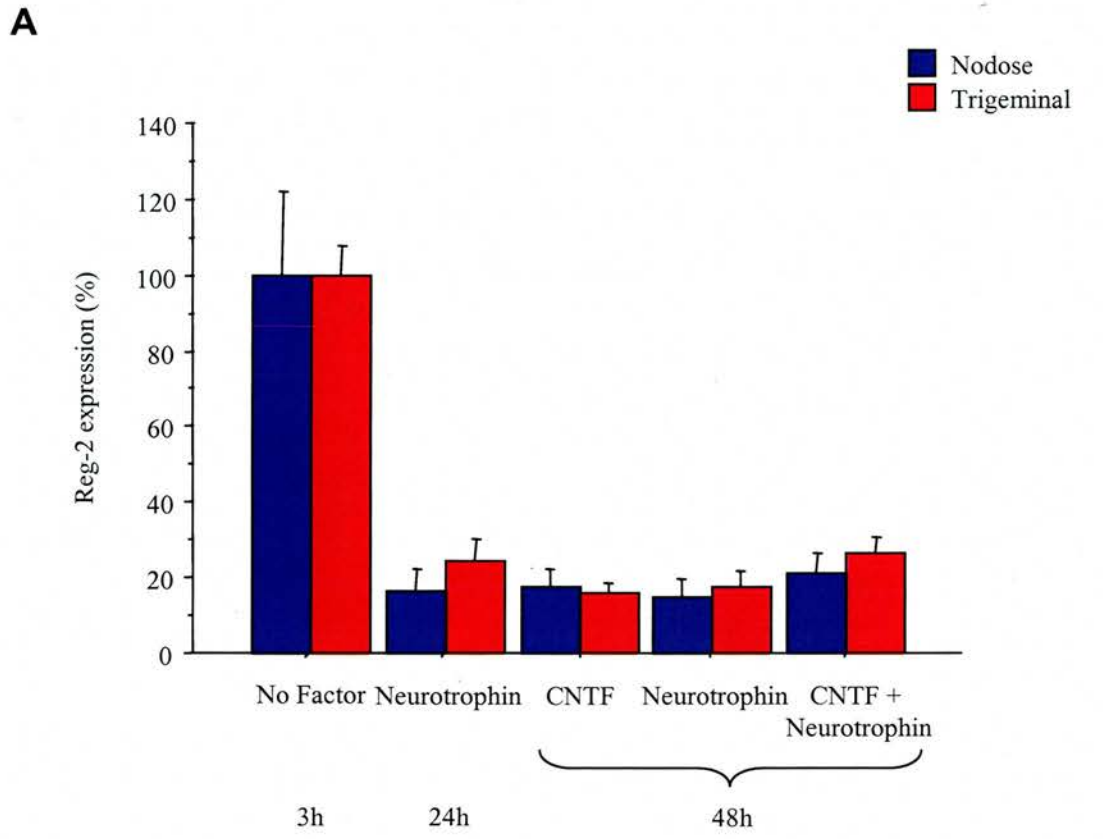
* **Figure 4.5** *Reg-2 expression is not rescued by CNTF stimulation after 24h cultured with neurotrophins.*
Purified P1 nodose and trigeminal neurons were cultured for 24 hours in the presence of BDNF (10 ng/ml, nodose neurons) or NGF (10 ng/ml, trigeminal neurons), washed and supplemented with CNTF (50 ng/ml), neurotrophin, or both for a further 48 hours. Reg-2 mRNA expression was quantified using competitive RT-PCR. A) Reg-2 mRNA expression. B) Percent survival. The levels of Reg-2 are expressed relative to the 0h control group to allow for comparison and the means and standard deviations of data obtained from 3 duplicate cultures are shown. Statistical comparisons shown are with respect to the 0h group, * $p<0.05$, ** $p<0.001$


A
Nodose
Trigeminal
Reg-2 expression (%)
140
120
100
80
60
40
20
0
No Factor
Neurotrophin
CNTF
Neurotrophin
CNTF + Neurotrophin
3h
24h
48h

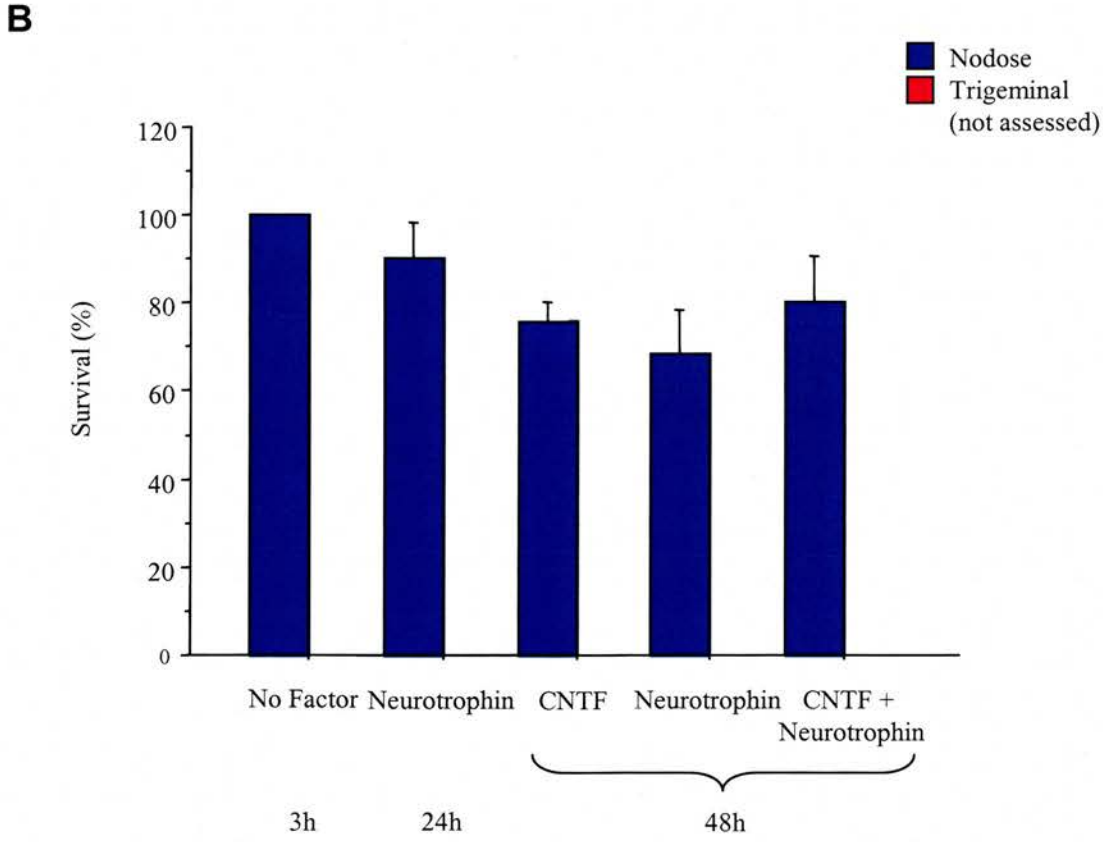

B
Nodose
Trigeminal
(not assessed)
Survival (%)
120
100
80
60
40
20
0
No Factor
Neurotrophin
CNTF
Neurotrophin
CNTF + Neurotrophin
3h
24h
48h

Figure 4.5

4.3.2. Stimulation of cultured neonatal neurons with Reg-2 results in Akt and Erk1/2 activation in nodose neurons, but only Erk1/2 activation in trigeminal neurons.

The receptor for Reg-2 has yet to be identified, therefore I could not address whether either nodose or trigeminal neurons have the potential to respond to Reg-2. I thus decided to look at whether Reg-2 was capable of inducing the phosphorylation of two intracellular kinases commonly involved in survival and neurite outgrowth, known biological effects of CNTF in nodose and trigeminal neurons. This was done by using immunoblotting techniques. P1 nodose and purified P1 trigeminal neurons were established in culture for three hours in the absence of trophic support, before being stimulated for 5, 15 or 30 minutes with 1 ng/ml Reg-2. These cultures were lysed immediately after stimulation and the lysate collected. The proteins in the sample were then electrophoretically separated on a non-denaturing polyacrylamide gel and transferred to a PVDF membrane. This was probed sequentially using phospho-Akt, phospho-Erk1/2 and pan-Akt antibodies. Figure 4.6 shows images of a single gel that has been probed with all three antibodies, and is representative of the results seen in three separate experiments. It shows that stimulation of nodose neurons with Reg-2 induces an increase in Akt phosphorylation that is detectable after 5 minutes stimulation, and which is maximal at 15 minutes after stimulation. An increase in Erk1/2 phosphorylation was also detected in nodose neurons as early as 5 minutes after Reg-2 stimulation, and which was still detectable after a 30 minutes exposure. In contrast Erk1/2 activation in trigeminal neurons was seen after 5 minutes stimulation with Reg-2, reaching a maximum after 15 minutes exposure, but no phosphorylation of Akt was detected at any time point. These results suggest that Reg-2 is capable of activating intracellular signalling pathways in both nodose and trigeminal neurons, and that the intracellular consequences of Reg-2 exposure in nodose and trigeminal neurons are overlapping but not identical.

4.3.3. Reg-2 promotes the survival of selected populations of sensory neurons during development

The observation that exposure of nodose or trigeminal neurons to Reg-2 resulted in the phosphorylation of Akt, a kinase commonly implicated in survival-related

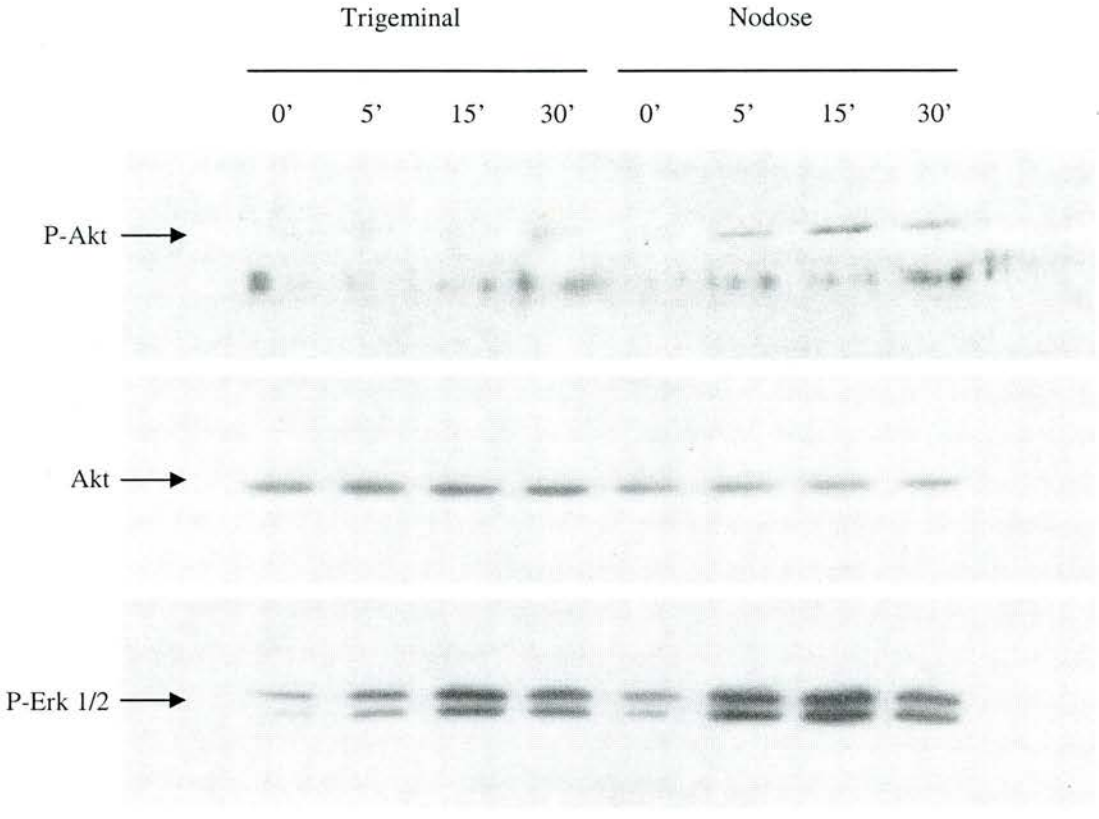


Figure 4.6 *Stimulation of cultured neonatal neurons with Reg-2 results in Akt and Erk1/2 phosphorylation in nodose neurons, but only Erk1/2 phosphorylation in trigeminal neurons.* P1 nodose and trigeminal neurons were cultured in the absence of trophic factors then stimulated for 5, 15 or 30 minutes with Reg-2. Here western blots were performed with the same membrane being probed for phosphorylated Akt and Erk1/2, as well as a control blot with a pan Akt antibody. These images are representative of three independent experiments.

signalling cascades (Alonzi et al., 2001; Crowder and Freeman, 1998; Downward, 1998; Klesse and Parada, 1998; Thompson et al., 2004; Virdee et al., 1999; Xue et al., 2000; Zhang et al., 2000)), in only one of two populations of sensory neurons known to survive in response to CNTF led me to investigate whether either neuronal population could survive when cultured in the presence of Reg-2. Low-density dissociated cultures of nodose and trigeminal neurons were established from mice at postnatal day 1 and supplemented with a range of doses of Reg-2. This age is equivalent to the age at which Reg-2 promoted CNTF-induced neuronal survival in developing motor neurons, as it is an age at which the later phases of neuronal death are occurring in these ganglia (Nishimune et al., 2000) The survival of these neurons was assessed after 48 hours in culture by counting the number of surviving neurons and expressing this as a percentage of the number of neurons attached to the dish three hours after plating. Figure 4.7 shows that in defined medium alone only 22% of nodose neurons and less than 3% of trigeminal neurons survive after 48 hours in culture. In nodose neurons concentrations of 0.01 ng/ml Reg-2 and over promote significant increases in neuronal survival, with concentrations above 0.1 ng/ml producing a maximal effect. Reg-2 did not promote the survival of neurons of the trigeminal ganglia at any concentration. This effect is consistent with my previous observation that Reg-2 induced Akt phosphorylation in nodose but not trigeminal neurons.

I also chose to compare Reg-2 induced neuronal survival to that promoted by other neurotrophic factors in both nodose and trigeminal neurons. Again dissociated cultures of these neurons from P1 mice were established and supplemented with a range of neurotrophic factors at saturating concentrations and survival assessed after a 48 hour incubation period. BDNF is a well characterised neurotrophic factor for nodose neurons, and in my cultures it aided 86% of the initially plated neurons to survive to 48 hours (10ng/ml), while only 31% survived without trophic support. (Figure 4.8A). The percentage of neurons surviving in the absence of trophic support is slightly improved in this culture compared to that in figure 4.7 because neurons generally survive better when cultured in the larger volume of media present in 35mm dishes, as compared to the 11mm wells. Reg-2 (0.1ng/ml) also promoted the

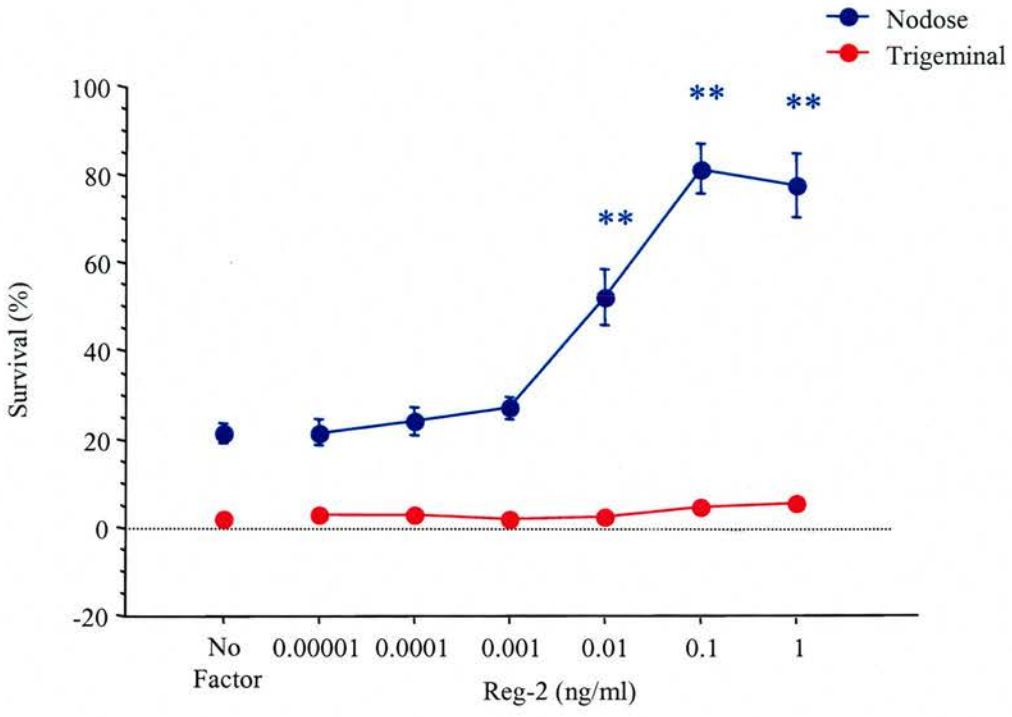


Figure 4.7 *Reg-2 promotes the survival of neonatal nodose, but not trigeminal, neurons.*
P1 nodose neurons were cultured in the presence of increasing concentrations of Reg-2 and their survival quantified 48 hours after plating. The means and standard errors of data obtained from three separate triplicate cultures for each experimental condition are shown. Statistical comparisons shown are with respect to untreated control neurons, * $p<0.05$, ** $p<0.001$

survival of 85% of nodose neurons after 48 hours in culture, and CNTF (50ng/ml) promoted the survival of a similar proportion of neurons (72%, no significant difference). Supplementing nodose neurons with Reg-2 and CNTF together produced no additional increase in survival, suggesting that Reg-2 and CNTF act on the same population of nodose neurons. Figure 4.8B presents a similar experiment performed in P1 trigeminal neurons. NGF, the neurotrophin known to promote the survival of trigeminal neurons, supported the survival of 61% of neurons after 48 hours in culture, and in the absence of trophic support just 16% survived. In trigeminal neurons both CNTF (50 ng/ml) and GDNF (10ng/ml) have been reported to support sub-populations of trigeminal neurons and in this experiment produced 42% and 26% survival, respectively ($p<0.001$ and $p<0.05$ w.r.t No Factor controls). Reg-2, as expected, did not promote the survival of P1 trigeminal neurons and, at a dose that promotes survival in nodose neurons, was unable to facilitate the neuronal survival induced by either CNTF or GDNF. Together these results show that Reg-2 effectively promotes the survival of neonatal nodose neurons, while having no survival promoting effects in trigeminal neurons at the same age.

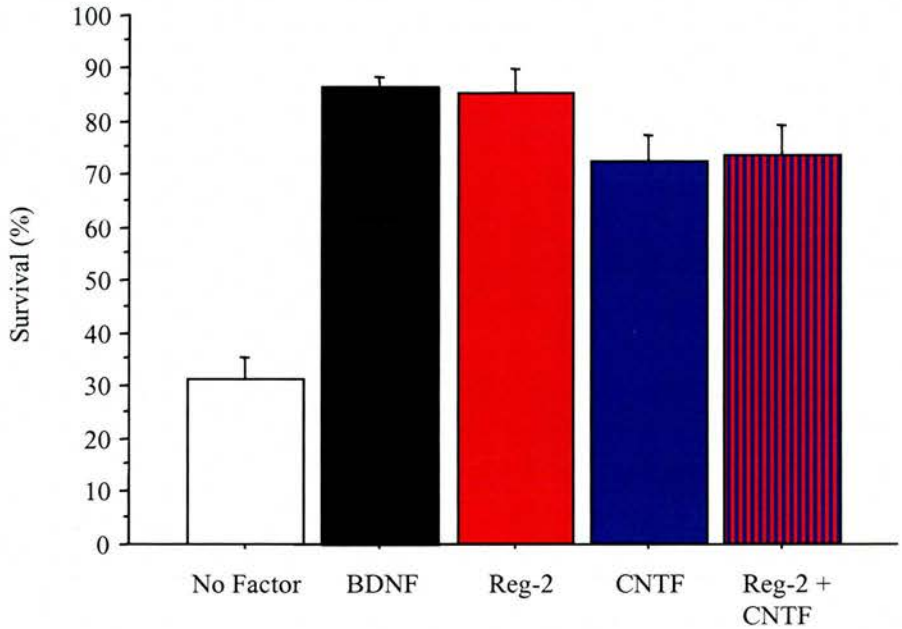
4.3.4. Reg-2 does not promote neurite outgrowth in trigeminal neurons

TheMAP kinase intracellular signalling pathway has been reported to be the principal pathway involved in signalling the promotion of neurite outgrowth, rather than neuronal survival, particularly in neonatal peripheral neurons (Aletsee et al., 2001; Aletsee et al., 2002; Alonzi et al., 2001; Choung et al., 2002; Creedon et al., 1996; Klesse and Parada, 1998; Sjogreen et al., 2000; Thompson et al., 2004;

Figure 4.8 *Reg-2 is as effective as neurotrophins or cytokines at promoting the survival of neonatal nodose neurons.*
P1 nodose and trigeminal neurons were cultured in the presence of Reg-2, neurotrophins, cytokines, or combinations thereof and their survival quantified 48 hours after plating. A) Survival of nodose neurons cultured in the presence of either BDNF (10 ng/ml), Reg-2 (0.1 ng/ml), CNTF (50ng/ml) or Reg-2 and CNTF together. B) Survival of trigeminal neurons cultured in the presence of NGF (10 ng/ml), Reg-2 (0.1 ng/ml), CNTF (50ng/ml), GDNF (10 ng/ml) or Reg-2 in combination with CNTF or GDNF. The means and standard errors of data obtained from three separate triplicate cultures for each experimental condition are shown.

A

Nodose



B

Trigeminal

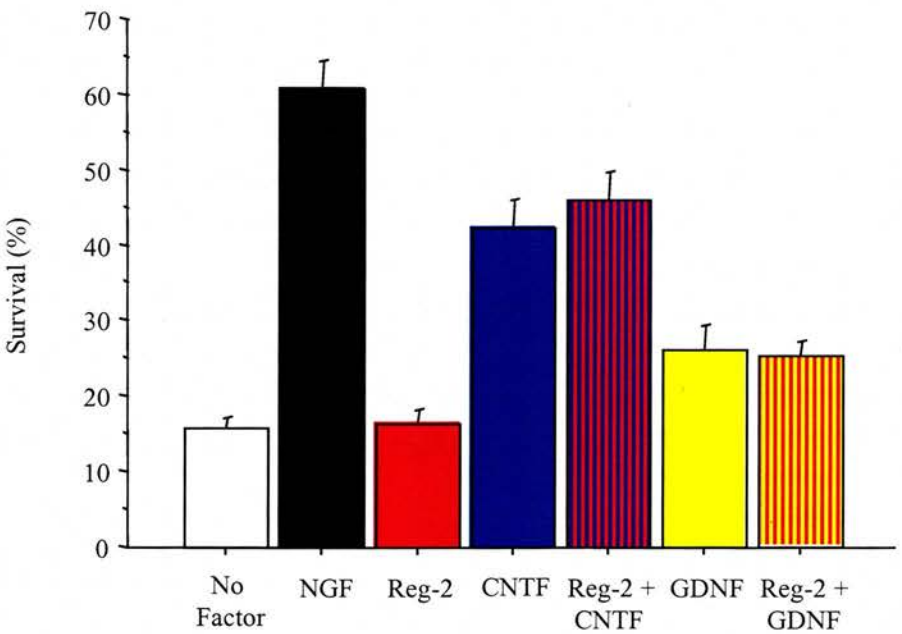


Figure 4.8

Virdee and Tolkovsky, 1996; Xue et al., 2000; Zhang et al., 2000). The activation of Erk1/2 in trigeminal neurons after Reg-2 stimulation therefore indicates that while Reg-2 is unable to promote the survival of these neurons it may regulate neurite outgrowth. To investigate this, I established low density cultures of P1 trigeminal neurons and incubated them in defined media containing either NGF alone (10ng/ml), NGF plus Reg-2 (1ng/ml) or NGF plus CNTF (50ng/ml). Trigeminal neurons could not be cultured with Reg-2 alone, due to the lack of survival effect. The outgrowth of these neurons was then studied after 24, 48 and 72 hours in culture.

Figures 4.9A, B and C show the Sholl analyses of neurite outgrowth after 24, 48 and 72 hours respectively. Trigeminal neurons cultured in NGF alone elaborated significant neurite arbours in the first 24 hours in culture. A mean of over 8 intersections are found at 50μm from the cell body, increasing to a peak of 13.2 intersections at 100μm from the cell body. At this time point addition of either Reg-2 or CNTF to the culture medium has no effect on the arbours elaborated by these neurons. After a further 24 hours in culture the neurite arbours of P1 trigeminal neurons cultured in the presence of NGF alone have increased in both size and complexity, and in the Sholl analysis the maximal number of intersections has increased to 26.2, occurring 150μm from the cell soma. The addition of Reg-2 to the culture medium had no effect on the pattern of neurite outgrowth, however supplementing the cultures with CNTF resulted in a significant change in morphology with a lower peak number of intersections (14.4) occurring further from the centre (250μm). By 72 hours after the addition of trophic factors this difference has disappeared and the neurons cultured under each condition assume a mean morphology closer to that of the neurons cultured with NGF plus CNTF at 48 hours, with a peak number of between 15.1 and 20.0 intersections occurring between 200

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* **Figure 4.9** *Reg-2 does not promote neurite outgrowth in neonatal trigeminal neurons*
P1 trigeminal neurons were cultured for 72 hours in medium supplemented with NGF (10ng/ml), NGF + Reg-2 (0.1 ng/ml) or NGF + CNTF (50 ng/ml) and the neurite arbour morphology assessed at 24, 48 and 72hours using the Sholl analysis and analysis of total neurite length and number of branch points. The means and standard errors of data obtained from 20 neurons in each experimental condition are shown, * $P<0.05$.

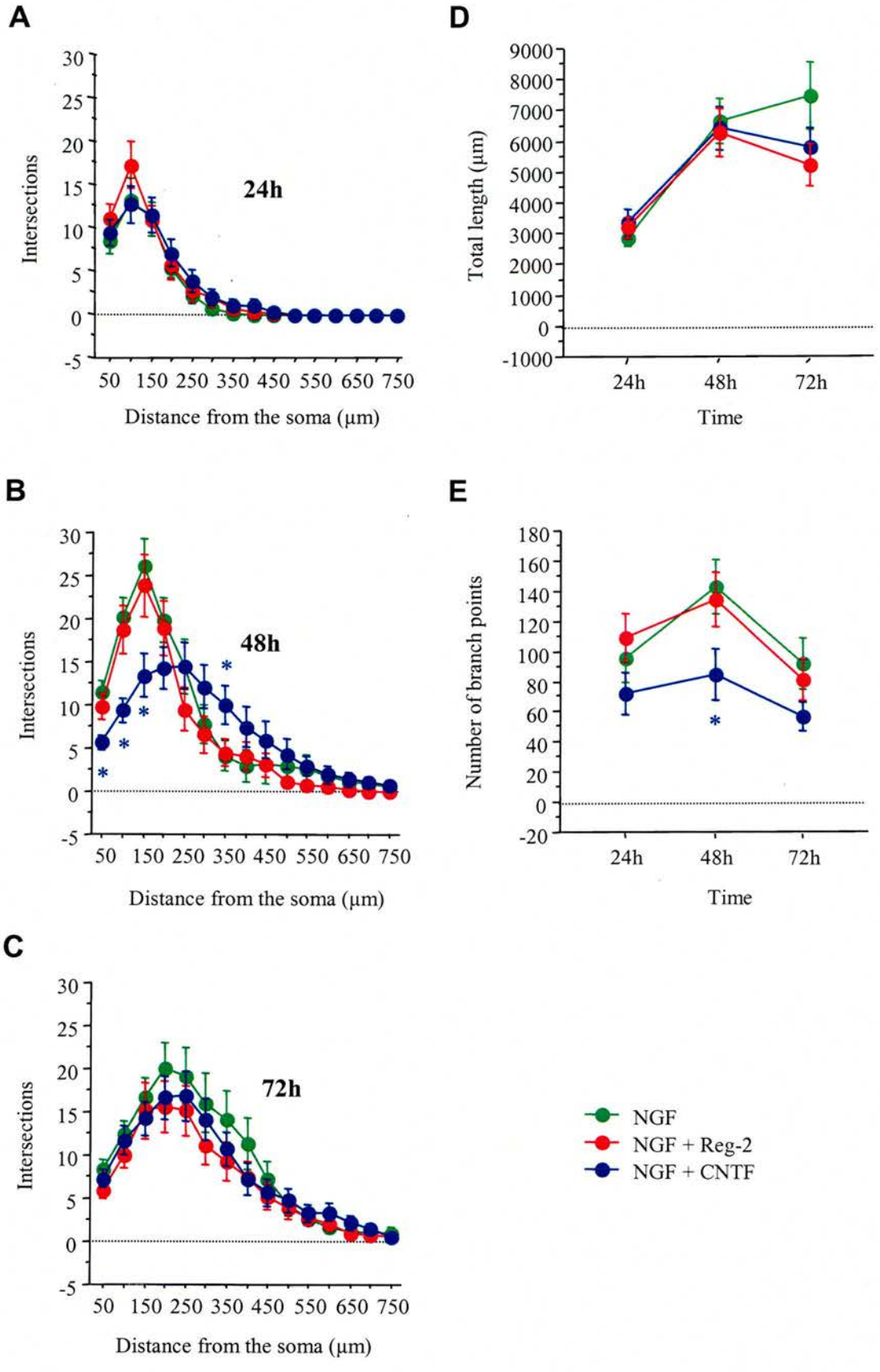

A
24h
Intersections
Distance from the soma (µm)
B
48h
Intersections
Distance from the soma (µm)
C
72h
Intersections
Distance from the soma (µm)
D
Total length (µm)
24h
48h
72h
Time
E
Number of branch points
24h
48h
72h
Time
NGF
NGF + Reg-2
NGF + CNTF

Figure 4.9

and 250 μm from the cell soma, suggesting that while CNTF may have promoted the rate at which the neurons assume a more mature neurite arbour, it had no long-term effect on the morphology of trigeminal neurons in culture. Analysis of total neurite length and branching reveals that while there is no difference in the total length of neurites between any factor combination at and time point, CNTF causes a significant decrease in the number of branch points after 48 hours in culture (figures 4.9D and E respectively). This suggests that the difference in the shape of the Sholl analysis is because the neurites are branching less, but growing further between each branch point. Together these results suggest that Reg-2 has no effect on neurite outgrowth in trigeminal neurons, however it should be noted that these results are only preliminary. Any repetition of these results was prevented by deterioration in the Reg-2 protein and our inability to obtain any additional protein that promoted the survival of nodose neurons.

4.3.5. Other Reg-family members are expressed in sensory ganglia during development

Reg-2 belongs to a family containing 6 different murine proteins, therefore one possibility is that another family member might play the role of the necessary signalling intermediate for cytokine-induced neuronal survival in trigeminal neurons that Reg-2 plays in motoneurons and possibly neurons of the nodose ganglia. To this end I studied the expression of the mRNAs coding for each of the family member in nodose and trigeminal neurons throughout development. Figures 4.10A and B show representative semi-quantitative RT-PCR gels for each family member in nodose and trigeminal ganglia respectively. In each gel two bands are seen, the upper band representing GAPDH mRNA expression and the lower band representing the relevant family member in all cases except for that of Reg-III□(Reg-2) where the bands are reversed and the upper band represents Reg-2 and the lower GAPDH. Reg-2 mRNA expression was included to validate this method of detection as compared to the competitive RT-PCR used earlier, and to calibrate the levels of primers used in the reaction. This is necessary as the levels of GAPDH mRNA expressed is approximately 250 times higher that those of reg-2. Increasing the excess of reg-2 primers relative to the levels of GAPDH primers increases the amplification rate of

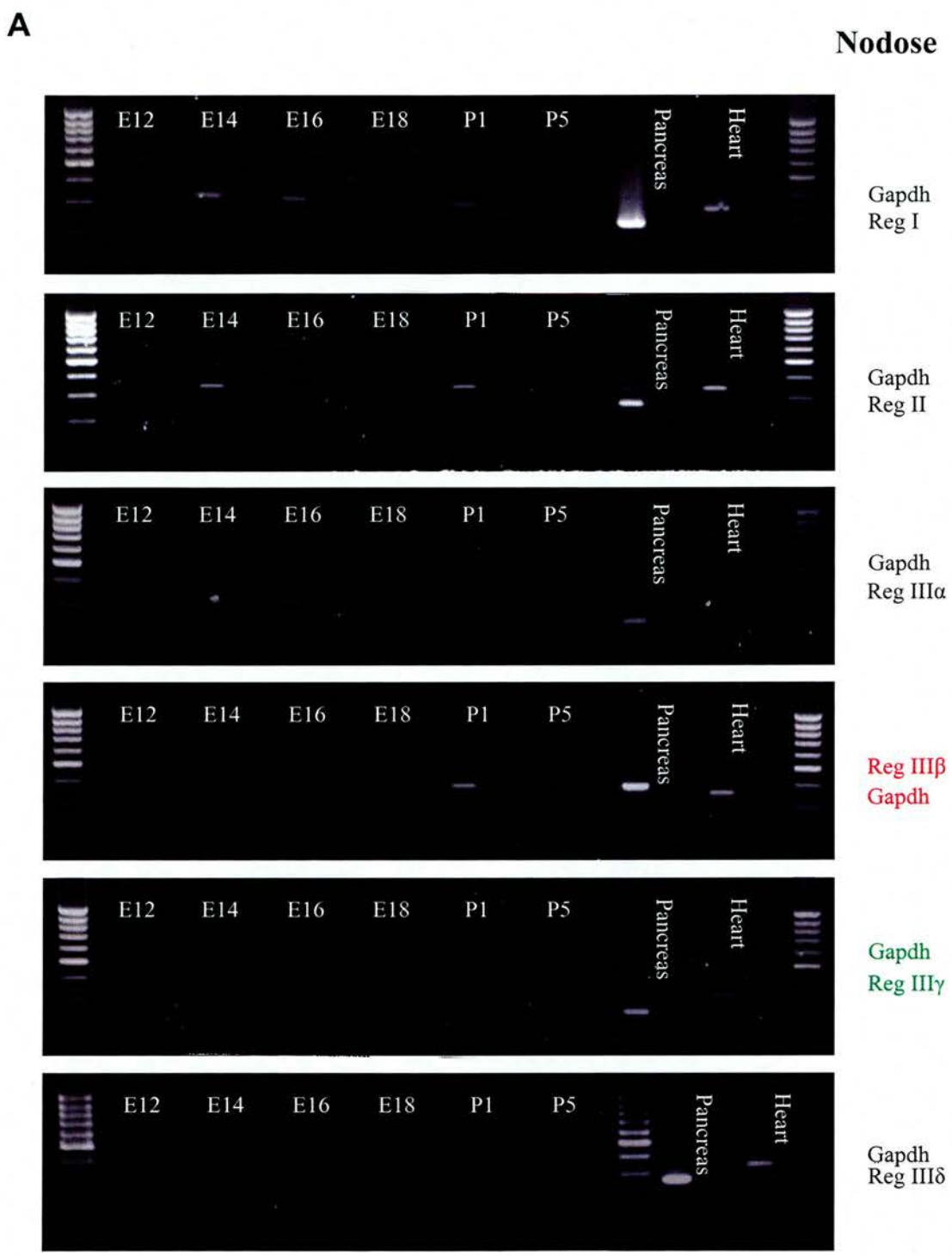


Figure 4.10 *Reg-IIIα and Reg-IIIγ mRNAs are expressed in sensory ganglia during development.* Nodose and trigeminal ganglia were dissected from mice between E12 and P5 and their expression of Reg family mRNAs assessed using semi-quantitative RT-PCR. A) Semi-quantitative RT-PCR gels of the expression of all Reg family genes in nodose ganglia. B) Semi-quantitative RT-PCR gels of the expression of all Reg-2 family genes in trigeminal ganglia. C) Quantification of the level of Reg IIIα expression in the nodose ganglia D) Quantification of the levels of Reg IIIα and Reg IIIγ expression in the trigeminal ganglia. The means and standard deviations of data obtained from 3 individuals at each age are shown. Statistical comparisons shown are with respect to E12 ganglia, * $p<0.05$, ** $p<0.001$

B

Trigeminal

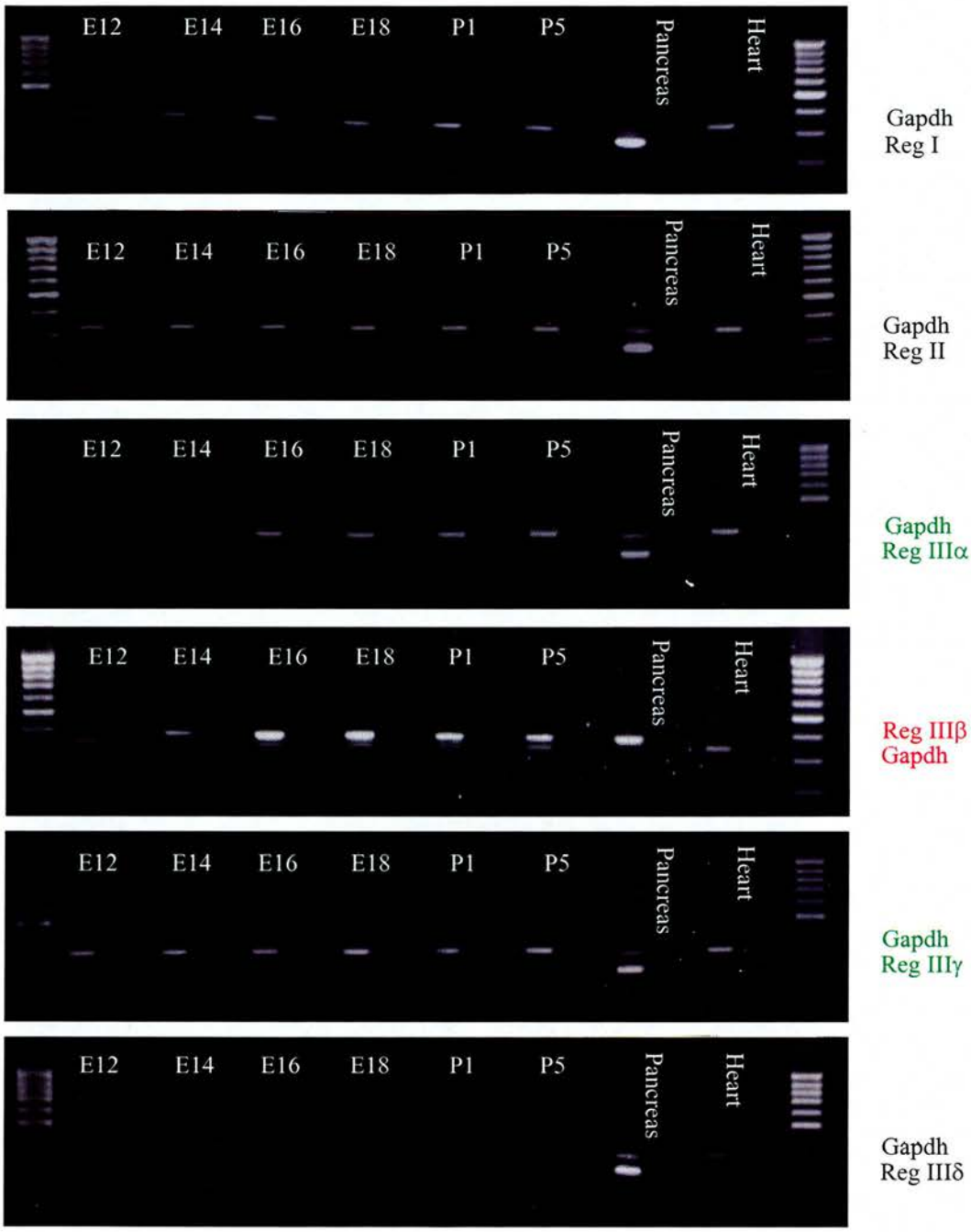
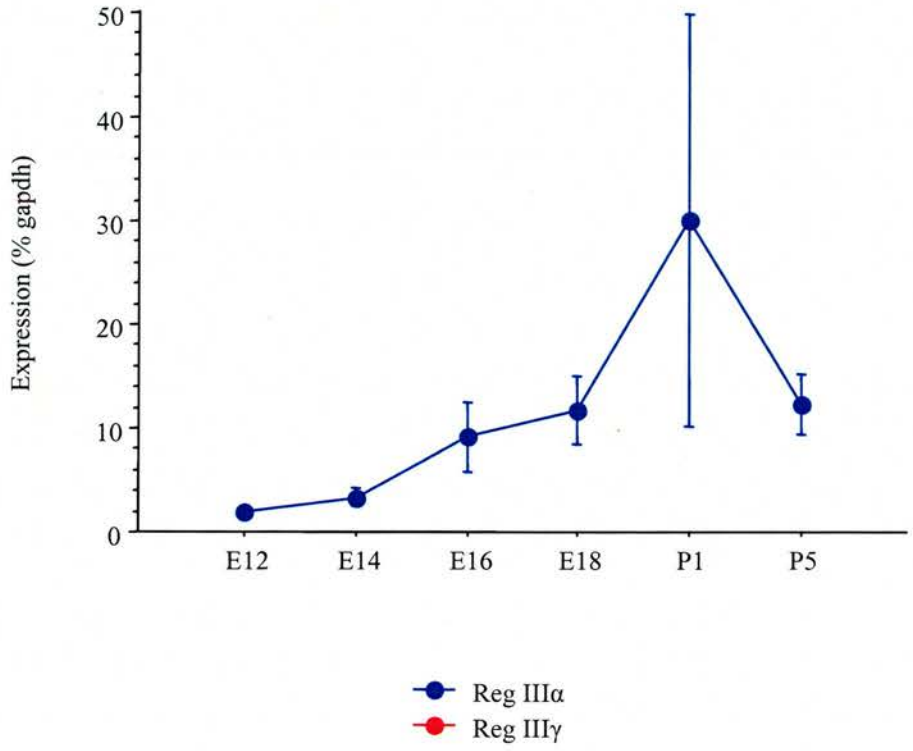


Figure 4.10

C

Nodose



D

Trigeminal

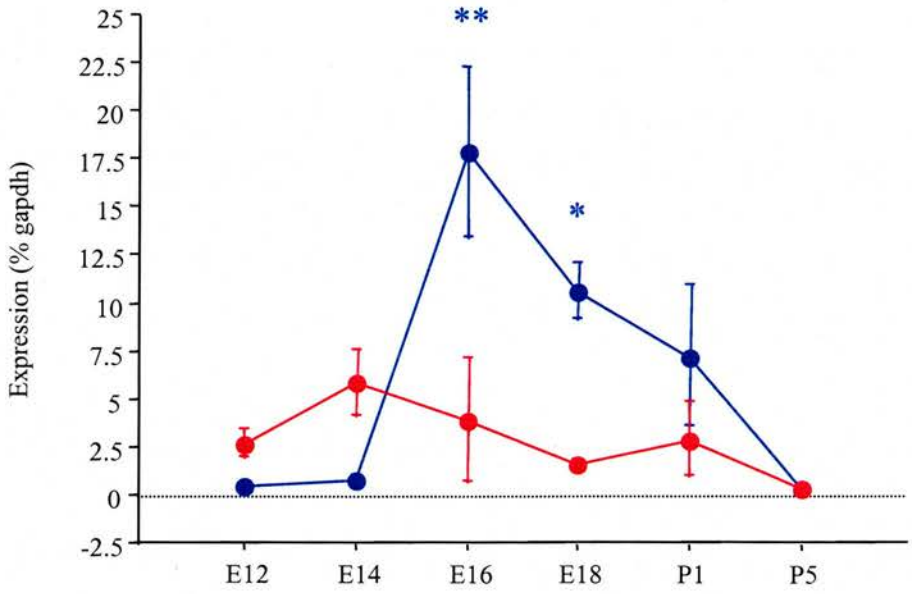


Figure 4.10

the Reg-2 reaction relative to the GAPDH reaction thus allowing both species to be detected in the same gel without one being overwhelmed by the other, and while still allowing the GAPDH levels to be used as a control for variation in the amount of between samples. In these reactions the reg family primer concentrations used are 5 times those of GAPDH. Reg-2 expression in this experiment followed a similar pattern observed in the quantitative experiments, with mRNA detected between E16 and P5 in the nodose ganglia and between E14 and P5 in trigeminal ganglia. In both ganglia the intensity of the reg-2 band is greater than or equal to that of the GAPDH band and reg-2 expression can also be detected in the pancreas, a tissue that has previously been reported to express all reg-family genes, and is undetectable in the heart, which was used as a negative control (Abe et al., 2000; Narushima et al., 1997). In the nodose ganglia (figure 4.10A) very little expression of mRNAs for other reg-family proteins could be detected, the only occurrence being the presence of a Reg-III□band at P1. In trigeminal ganglia the situation appears a little different, with bands representing Reg-III□being detectable between E16 and P1 and Reg-III□ bands being observed at E14 and P1. In all cases the integrity of the reaction was confirmed by the presence of a band of the predicted size in the positive control reaction (pancreas) and its absence in the negative control (heart).

The semi-quantitative RT-PCR for the genes where any positive identification of expression occurred was repeated using three samples at each age and the results quantified by expressing the intensity of the mRNA band for the gene of interest as a percentage of the intensity of the GAPDH band, as quantified using the Phoretix 1D analysis software. The results of these experiments can be seen in figures 4.10C and D for the nodose and trigeminal ganglia, respectively. In the nodose ganglia, apart from Reg-2, only the expression of Reg-III□was detected in the initial experiments. Quantification of this expression throughout development shows that low levels of reg-III□expression could be detected from E16 onwards, but this expression was variable, particularly in the samples studied at P1 where one sample had high levels of reg-III□and the others had very low levels. These levels appear to be much lower than the levels of Reg-2 expression would be if quantified, as in figure 4.10A the intensity of the Reg-2 bands in mice at E16 or older are either the same as or greater

than those of GAPDH, whereas when reg-III□expression was quantified, the intensity of the bands appeared to be around 10% of that of GAPDH. In the trigeminal ganglia, expression of reg-III□and reg-III□were initially detected and selected for further analysis. Significant increases in reg-III□expression could be detected in trigeminal neurons between E16 and E18 ($p<0.001$ and $p<0.05$ respectively, relative to E12 and E14), with levels decreasing to nothing by P5. As with the nodose ganglia, these levels of expression were very low compared to those of Reg-2, where in the trigeminal ganglia the intensity of the Reg-2 band appeared much greater than that of GAPDH between E14 and P5. Quantification of the levels of reg-III□expression in the trigeminal ganglia revealed a very low level of expression with no significant increase in expression at any age. Together these results suggest that there might be some developmental pattern in the expression of Reg-III□, particularly in the trigeminal ganglia.

4.4. Discussion

This study has investigated the relationship between Reg-2 and CNTF in promoting the survival and outgrowth of sensory neurons from the developing peripheral nervous system. Reg-2 mRNA expression was detected in both nodose and trigeminal ganglia *in vivo* during a defined developmental window, from E16 to P1. Reg-2 has previously been shown to be developmentally regulated, both in the nervous system and elsewhere, with its expression occurring during periods when cells are subject to stress, such as when neuronal number is adjusted to the size of target during the development of the nervous system (Livesey et al., 1997; Nishimune et al., 2000) or during weaning in the gastrointestinal epithelia (Chakraborty et al., 1995). Detection of Reg-2 in purified neonatal nodose and trigeminal neurons confirmed that it is expressed at least in part by the neurons of these sensory ganglia. Reg-2 expression occurred in both populations of sensory neurons during the later stages of establishment of innervation and programmed cell death, starting soon after the peak levels of neuronal apoptosis are seen (Davies and Lumsden, 1984a; Pinon et al., 1996). It is likely that the exposure of neurons to novel target-derived trophic factors, such as cytokines, induces Reg-2 expression in these neurons. However, the cause of the subsequent fall in Reg-2 expression soon after birth may not be a result of decreased cytokine expression at this time. Murphy et al (1993) found increased LIF mRNA expression in a range of DRG neuron target fields, including skin and muscle, at E12, just prior to when these neurons start responding to LIF *in vitro*. In some tissues LIF appears to be transiently expressed, while in others expression is over a much longer term, well into the postnatal period (Murphy et al., 1993; Yamamori, 1991). No information about cytokine expression is available for the target fields of nodose and trigeminal neurons, however, if they were continuing to have contact with LIF postnatally, an alternative mechanism must be present to suppress Reg-2 expression. In the liver, normal cells have been shown to express a transacting factor that binds to a silencing element in the Reg-2 promoter and which is absent in hepatoma cells that can express Reg-2 protein (Dusetti et al., 1996). Such an intrinsic regulatory mechanism could limit the duration of Reg-2

expression after initial exposure to cytokines, alternatively another external factor could block Reg-2 mRNA expression after a certain time period.

There is currently no direct evidence of a link between neurotrophins and Reg-2 expression. However crosstalk between neurotrophin and cytokine signalling pathways in neuronal systems has been reported. In neuroblastoma cell lines NGF modulates CNTF-induced neuropeptide expression (Symes et al., 1993), which, like Reg-2 expression, requires STAT activation (Symes et al., 1994). Also, culturing adult SCG explants for several days in the presence of either NGF or BDNF results in decreased STAT activation after 5 days, compared to untreated ganglia where STAT activation continues for up to 8 days *in vitro*. This suggests that these neurotrophins limit long-term STAT activation (Rajan et al., 1998). The serine phosphorylation inhibitor H7 blocked this effect. In some cases serine phosphorylation has been shown to be necessary for maximal STAT activation (Chen et al., 2004; Ng and Cantrell, 1997; Wen et al., 1995; Zhang et al., 1995a). However Jain et al. (1998) and Chung et al. (1997) have shown that in different circumstances serine phosphorylation by ERK2 was enough to decrease STAT-3 activation. This suggests that neurotrophins could block STAT activation by activating an inhibitory serine kinase upstream of STAT. Several protein groups negatively regulate STAT-activation in a variety of manners. These include protein phosphatases, such as SHP1 and 2, PIAS (Protein Inhibitors of Activated STAT) proteins and SOCS (Suppressor of Cytokine Signalling; (Chen et al., 2004; Wu et al., 2002). SHP-2 regulates the dephosphorylation of STAT-1 in A431 cells and is also activated by neurotrophin signalling, when it mediates survival and neurite growth (Araki et al., 2000b; Araki et al., 2000a; Chen et al., 2002; Ohnishi et al., 1999; Okada et al., 1996; Takai et al., 2002; Wu et al., 2002). SOCS protein expression is regulated by STAT activation itself therefore SOCS proteins act as part of a negative feedback loop controlling JAK/STAT signalling. These proteins act by inhibiting JAK kinase and thus STAT phosphorylation (Endo et al., 1997; Hilton, 1999; Nicholson et al., 1999; Starr et al., 1997). SOCS expression can be regulated by the growth factor FGF-2 during development, suggesting its expression can be regulated by other signalling pathways such as MAPK or PI3K (Turnley and Bartlett, 2000). SOCS-3 expression has also

been found to be regulated by NF□B in fibroblasts (Li et al., 2002). Additionally, as I discussed in chapter three, not all cytokine signalling is dependent on a STAT related pathway. One or more of these proteins are potential mediators of crosstalk between neurotrophin and cytokine signalling pathways. In chapter 3, I also discussed that the physiological outcomes of cytokine exposure is dependent upon a balance between STAT and MAPK signalling (Bonni et al., 1997; Ihara et al., 1997; Marz et al., 2002; Turnley and Bartlett, 2000). The presence of neurotrophins in a culture system, which can themselves activate MAPK signalling, can therefore affect the outcome of cytokine exposure and could potentially affect Reg-2 expression through alterations in this balance (Ihara et al., 1997; Turnley and Bartlett, 2000).

Since neuronal expression of Reg-2 mRNA is largely dependent on functional STAT-3 in both sensory neurons during development (this chapter) and in motor neurons after axotomy (Schweizer et al., 2002), it is likely that any effect of neurotrophins on cytokine-induced STAT-3 activation also impact on Reg-2 expression. The similarities in the duration of expression of Reg-2 during development and the delay before neurotrophins suppress STAT-3 activation in organotypic cultures (Rajan et al., 1998) provides a clue, suggesting that this might be the case, although how such a delay occurs is unknown. In dissociated culture, the expression of Reg-2 mRNA by peripheral sensory neurons was maintained for at least 24 hours by the presence of IL-6 type cytokines in the culture medium, but not by the presence of neurotrophins. Unexpectedly, when neurons were cultured with a combination of both cytokine and neurotrophin together, differences between the responses of the two populations were detected. Culturing trigeminal neurons in the presence of cytokine and NGF maintained Reg-2 mRNA expression at 24 hours, while in nodose neurons exposed to cytokine and BDNF there was a large drop in the level of Reg-2 expression after 24 hours. This raises several questions, the principal of which is whether CNTF is indeed promoting the expression of Reg-2 mRNA in either population of neurons. Exposure of both types of neurons to concentrations of cytokines that have been established in our lab as eliciting a maximal survival response (Horton et al., 1998) did not promote increased expression of Reg-2 mRNA, although the levels of expression seen in the presence of cytokines were

significantly greater than that seen with neurotrophins at the same time point. One explanation is that Reg-2 mRNA expression in these neurons is already maximal at the time of dissection, although this might imply that saturating levels of CNTF are present in vivo, a situation which would contradict the prevailing theory that it is limited quantities of neurotrophic factors that select cells for survival or death at this period of development. Alternatively Reg-2 mRNA expression in these neurons could be entirely "constitutive", controlled by an as yet uncharacterised mechanism. If this is the case, the decreased Reg-2 mRNA expression seen in the presence of neurotrophins may well be an active process, a conclusion that would be supported by the effect seen in the presence of cytokine plus neurotrophin in nodose but not trigeminal neurons. The results seen in trigeminal neurons suggest that NGF does not affect Reg-2 mRNA expression since this remains high when neurons are cultured in the presence of both factors. Currently the weight of published evidence in both neuronal and non-neuronal cells, particularly the abolition of Reg-2 expression in LIF receptor and STAT-3 knockout mice (Livesey et al., 1997; Schweizer et al., 2002), suggests that it is most likely that cytokines actively maintain neuronal expression of Reg-2 mRNA in both populations. As for neurotrophins, my results suggest that BDNF, at least, is in some way implicated in regulating reg-2 mRNA expression. As I described earlier, despite no direct evidence relating neurotrophins to Reg-2, a mechanism can be postulated involving the crosstalk between neurotrophin and cytokine signalling pathways regulating levels of STAT-3 activation and subsequent gene expression. Alternatively neurotrophins could act to influence Reg-2 expression at a point less directly dependent on cytokine signalling. TNF-□has been shown to induce PAPI/Reg-2 expression via MEK1 (Malka et al., 2000). As both Reg-2 and BDNF have been show to activate the MAPK pathway, it is possible that there is an alternative pathway through which neurotrophins, or even Reg-2 itself, could influence Reg-2 levels, although in both cases a positive feedback loop, supporting Reg-2 expression, might be expected.

Additional experiments were performed to investigate whether re-exposure to cytokines could rescue Reg-2 mRNA expression after a 24h incubation in the presence of neurotrophins. No recovery of Reg-2 mRNA expression at 48 hours

occurred in any condition, however this may have been a consequence of inadequate experimental design. After 48h in culture these neurons are approaching the time at which Reg-2 expression is down-regulated *in vivo*. While in culture many external developmental influences are removed, neurons in my culture system may still execute an intrinsic program that terminates Reg-2 expression. Further experiments could clarify the roles of both neurotrophins and cytokines in the regulation of Reg-2 expression in peripheral sensory neurons. Evaluation of whether the levels of Reg-2 mRNA expression are maintained after continuous exposure to cytokines for 48 hours could be informative, as would evaluation of expression levels when neurons are maintained in culture not through neurotrophic support, but by inhibition of the apoptotic machinery. Following these studies the effect of switching between cytokines and neurotrophins could be re-examined. I would also like to have studied whether Reg-2 itself is capable of regulating its own expression in light of the fact that I have found that Reg-2 is capable of activating the MAP kinase pathway, a pathway implicated in TNF□-induced PAP1/Reg-2 expression in pancreatic acinar cell lines (Malka et al., 2000). This, however, was not possible because I was unable to obtain further supplies of functional Reg-2 protein.

Having detected the presence of Reg-2 in the peripheral sensory nervous system during a developmentally restricted period corresponding to the period during which Reg-2 promotes neuronal survival in other neuronal populations, I investigated whether Reg-2 could directly promote the survival of peripheral sensory neurons. In nodose neurons this was the case, however in P1 trigeminal neurons Reg-2 had no survival effects. This difference in the effect of Reg-2 on the survival of these neurons was surprising since CNTF promotes the survival of both nodose neurons and a substantial sub-population of trigeminal neurons at this age (Horton et al., 1998). Akt is a protein kinase that is widely reported as being involved in signalling neuronal survival in response to a variety of neurotrophic factors including cytokines (Brunet et al., 2001; Middleton et al., 2000; Nishimune et al., 2000). It is activated by phosphorylation therefore I investigated whether Reg-2 stimulation was capable of inducing Akt phosphorylation. In line with the survival experiments I found that in nodose, but not trigeminal neurons, a 15 minute exposure to Reg-2 induced robust

Akt phosphorylation. Reg-2 expression in nodose neurons is dependent on STAT-3 signalling, which in turn can be activated by cytokines, and in culture Reg-2 promotes neuronal survival to a similar extent as CNTF. Thus, while it is possible that cytokines exert their survival effect via the Reg-2 secreted signalling intermediate in nodose neurons this does not seem to be the case for trigeminal neurons. It should also be noted that while Reg-2 expression in nodose neurons first occurs at significant levels at E18, both CNTF and LIF have previously been found to promote the survival of a proportion of nodose neurons as early as E11. This suggests that at this early stage a Reg-2 independent mechanism mediates cytokine-induced survival. Likewise, there appears to be an alternative Reg-2 independent mechanism mediating cytokine-induced neuronal survival in trigeminal neurons. During the same developmental window, trigeminal neurons express Reg-2 more robustly that nodose neurons, yet Reg-2 does not promote survival in these neurons, despite their response to CNTF.

While Reg-2 does not promote the survival of trigeminal neurons, it stimulates ERK1/2 phosphorylation. ERK1/2 forms part of the MAP kinase cascade, a signalling pathway known to be important in the promotion of neurite outgrowth by a range of neurotrophic factors, including cytokines, in many neuronal types (Aletsee et al., 2001; Choung et al., 2002; Ihara et al., 1997; Marz et al., 2002; Sjogreen et al., 2000; Thompson et al., 2004; Wiklund et al., 2002; Wiklund and Ekstrom, 2000). Reg-I□and Reg IV have, to date, been reported to activate p38 MAPK signalling pathways (Hartupee et al., 2002), but no links have been described between any Reg protein and Erk1/2. I detected increased levels of phosphorylated Erk1/2 in both nodose and trigeminal neurons and, although not documented, saw robust neurite outgrowth in nodose neurons cultured in the presence of Reg-2. An effect of Reg-2 on neurite outgrowth in trigeminal neurons cannot be easily observed, as trigeminal neurons cultured in the presence of Reg-2 do not survive. A preliminary study was performed to investigate whether Reg-2 was capable of increasing neurite outgrowth in NGF-supplemented cultures of trigeminal neurons. In this study, Reg-2 appeared to have no ability to alter NGF-induced neuronal morphology, despite CNTF increasing the rate at which these neurons achieved a more mature neuritic arbour.

This observation does not completely exclude Reg-2 having a neurite outgrowth-promoting effect. NGF already promotes a significant amount of neurite outgrowth in trigeminal neurons and in other peripheral ganglia this activity has been reported to be through a MAP kinase-dependent mechanism (Liu and Snider, 2001; Pang et al., 1995a; Pang et al., 1995b; Xiao and Liu, 2003). It may therefore be that the effects of NGF on neurite outgrowth was masking a possible effect of Reg-2 on growth or that NGF was promoting maximal neurite outgrowth via the MAP kinase pathway. An alternative pathway could then mediate the effect of CNTF on neurite branching. If further Reg-2 protein was available for experimentation, culturing trigeminal neurons with a sub-saturating concentration of NGF, in combination with Reg-2, might reveal an effect of Reg-2. Another possibility would be to used caspase inhibitors or over-expression of an anti-apoptotic Bcl-2 family protein to maintain the neurons in culture with Reg-2 alone (Doxakis et al., 2004; Middleton and Davies, 2001; Werth et al., 2000).

The production of Reg-2 by developing trigeminal neurons might have a role in stimulating the proliferation of Schwann cell precursors, as it has been shown that Reg-2 promotes the division of dedifferentiated adult Schwann cells after nerve injury (Livesey et al., 1997). There are, for example, many more non-neuronal cells present in the trigeminal ganglia at this stage of development than in the nodose ganglia. The expression of Reg-2 between E16 and P1 also coincides with the period during which immature Schwann cells emerge, starting between E12 and E16 and continuing until the first postnatal days, after which they withdraw from the cell cycle and establish their mature phenotype (Dong et al., 1999; Syroid et al., 1996).

The difference in the effect of Reg-2 on the survival of newborn nodose and trigeminal neurons, both of which survive in response to CNTF suggests that Reg-2 is not a universal intermediate in CNTF-induced neuronal survival. There are two possibilities to explain this observation. Firstly, CNTF could activate pathways promoting neuronal survival and neurite outgrowth that are entirely independent of reg family proteins. This could include activation of MAPK pathways, as discussed in this chapter and in chapter 3 or through the expression of other STAT-induced genes. Genes containing STAT-binding elements and which are expressed in

response to STAT activation include other growth factors, such as HGF and OSM, as well as Bax, Bcl-2 and Bcl-xL (Catlett-Falcone et al., 1999; Dumon et al., 1999; Fukada et al., 1996; Kirito et al., 2002; Seto et al., 1988; Silva et al., 1999; Socolovsky et al., 1999; Tomida and Saito, 2004). Alternatively STAT activation by neuropoietic cytokines could promote the expression of a different member of the Reg family. All Reg family members carry IL-6 response elements in their promoter regions, meaning that activation of STAT transcription by neuropoietic cytokines could subsequently promote their expression. There is, as yet no information concerning whether any of these proteins have biological effects in neurons, although several are known to promote survival in other systems (see 1.4.3). To this end I examined the developmental expression of the other Reg family proteins in both nodose and trigeminal neurons. Most other Reg family mRNAs were not expressed in either nodose or trigeminal neurons, with only group III mRNAs present in either ganglion. Some expression of Reg-III□was detected in nodose ganglia, and both Reg-III□and reg-III□mRNAs were found in the trigeminal ganglia. In all cases, however, the levels detected in each ganglion were very low compared to those of Reg-2 and for Reg-III□in the nodose ganglia and Reg-III□in the trigeminal ganglia expression was not detected in all samples at a particular age. Reg-III□, in the trigeminal ganglia, was the only gene expressed robustly enough to suggest it might be physiologically relevant. Reg-III□expression was also developmentally restricted in a similar manner to Reg-2, between E16 and P1. The levels, compared to those of Reg-2 in the same ganglia, were low, however the levels of Reg-2 expression detected through competitive RT-PCR in the nodose ganglia, where Reg-2 appears to be biologically active, were also much lower than those expressed in the trigeminal ganglia. Only quantification of the absolute amounts of Reg-III□mRNA present in the trigeminal ganglia could allow better comparison. It might be of future interest to investigate whether Reg-III□has any physiological effect on trigeminal neurons.

The results presented in this chapter suggest that the relationship between Reg-2 and CNTF in sensory neurons is more complex than that described in motor neurons. In both the nodose and trigeminal ganglia CNTF appears to support Reg-2 expression in a STAT-3 dependent manner, consistent with published literature. My expression

studies suggest, however, that neurotrophins may also play a role in modulating Reg-2 expression. Additional evidence also suggested that Reg-2 is not an essential intermediate in all the physiological effects mediated by neuropoietic cytokines. There is also a possibility that Reg-2 may act to promote neurite outgrowth in trigeminal neurons, as it does in nodose neurons, but despite seeing phosphorylation of Erk1/2 in these neurons, I found no further evidence for such an effect. Due to the lack of functional Reg-2 protein I was not able to investigate any direct link between Reg-2 and NF□B in this system. Reg-2 has been reported to activate NF□B in motor neurons, and it was predicted that this would be through the classical pathway for NF□B activation, that is via Akt and PI3K leading to phosphorylation and degradation of the NF□B inhibitory complex. In nodose, but not trigeminal neurons, Reg-2 induced Akt phosphorylation. This is consistent with the survival studies and indicates that a signalling pathway through Akt could well be a responsible for Reg-2-induced neuronal survival. In the previous chapter, however, I found that overexpression of superrepressor I□B□did not affect CNTF-induced survival of nodose neurons suggesting that either Reg-2 does not induce neuronal survival via NF□B, or that if it does that Reg-2 may not be an intermediate in CNTF-induced neuronal survival. The observation that Reg-2 also promotes neurite outgrowth in nodose neurons suggests that Reg-2 is unlikely to be a point at which CNTF signalling for survival and neurite outgrowth diverges. Interestingly, the range of ages over which Reg-2 is potentially produced by both nodose and trigeminal neurons, as deduced from express studies of Reg-2 mRNA is the same period during which I found neurite outgrowth in nodose neurons to be dependent on NF□B signalling.

Conclusion

In this thesis I have investigated the role of NF□B-dependent gene expression in the development of the murine peripheral nervous system. In sensory neurons of the nodose ganglia I found that interfering with the NF□B signalling pathway, using a variety of chemical and genetic methods caused a substantial decrease in the size and complexity of the neurite arbors of neurons cultured in the presence of BDNF, without affecting neuronal survival. This effect was seen during a restricted developmental window, between E18 and P1, a period during which, *in vivo*, developing neurons are modifying and refining their process morphology and interconnectivity. In the same type of neurons, NF□B was also implicated in the neurite outgrowth of neurons cultured in the presence of CNTF, but not LIF.

I also investigated the role of NF□B-dependent gene expression in the elaboration of neurites from sympathetic neurons of the superior cervical ganglion. In this neuronal population, the study of neurite outgrowth is complicated by the role of NF□B in mediating neuronal survival. This was overcome through the use of caspase inhibitors to prevent cell death, and revealed that NF□B also plays a role in regulating the growth and complexity of neurite arbors in SCG neurons cultured in the presence of NGF. Together the studies in the nodose ganglia and SCG revealed a novel role for NF□B-dependant gene expression in the growth and elaboration of neurite arbors in the peripheral nervous system.

The final set of investigations that I performed for this thesis was investigating the role of Reg-2 in CNTF-induced survival of sensory neurons. Many of these studies were inconclusive, however my studies showed that Reg-2 is capable of promoting the survival of nodose, but not trigeminal neurons and that Reg-2 expression in both ganglia is dependent on the transcription factor STAT-3. Theses results suggest that Reg-2 is unlikely to be a universal intermediate in cytokine-induced neuronal survival, as had previously been suggested.

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Appendix 1: Abbreviations

AD	Alzheimers disease
BDNF	Brain derived neurotrophic factor
BMP	Bone morphogenetic protein
CAMK	Ca^{2+}/calmodulin-dependent protein kinase
cIAP	cellular inhibitor of apoptosis-1
CLC	Cardiotrophin-like compound
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CNTFRα	CNTF receptor α
COX	Cyclooxygenase
CREB	cAMP-responsive element-binding protein
CT-1	Cardiotrophin-1
DRG	Dorsal root ganglion
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
FGF	fibroblast growth factor
GDNF	Glial-cell line derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GFR (α3)	GDNF family receptor (α3)
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GPA	Growth-promoting activity
GSK	Glycogen synthase kinase
HGF	Hepatocyte growth factor
HIP	gene expressed in hepatocellular carcinoma-intestine-pancreas
ICAM	intercellular adhesion molecules
IFNγ	Interferon-γ
IKK	IκB kinase
IL-6	Interleukin-6
INGAP	Islet neogenesis-associated protein
iNOS	inducible nitric oxide synthase

IRE	IL-6-response element
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LIF	Leukaemia inhibitory factor
LIFRβ	LIF receptor β
MAGE	melanoma antigen
MAP	Microtubule associated protein
MAPK	Mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
MSK	mitogen- and stress-activated protein kinases
MSP	Macrophage-stimulating protein
NADE	P75 NTR-associated cell death executor
NAK	NFκB-activating kinase
NEMO	NFκB essential modifier
NFκB	Nuclear factor κB
NGF	Nerve growth factor
NIK	NFκB-inducing kinase
NP	Neuropoietin
NRAGE	Neurotrophin receptor-interacting MAGE
NRIF	Neurotrophin receptor-interacting factor
NT	Neurotrophin
OSM	Oncostatin M
PAP	Pancreatitis-associated protein
PDK	phosphoinositide-dependent kinase
PI3K	Phosphotidylinositol 3 kinase
PKA or PKC	Protein kinase A or C
PLC	Phosholipase C
PNS	Peripheral nervous system
PSP	Pancreatic stone protein
PTP	Pancreatic thread protein
RSK	ribosomal S6 kinase

SCG	Superior cervical ganglion
SH2	Src homology 2
SHP	SH2-containing tyrosine phosphatase
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
TAK	TGFβ-activating kinase
TNFα	Tumour necrosis factor α
TRAF	TNF receptor associated factor α

Appendix II: Supplementary Methods

Solutions and media used in tissue culture

0.1% Trypsin: 50mg of trypsin was added to 5ml of CMF-HBSS (Hanks' Balanced Salt Solution without calcium and magnesium, GibcoBRL) and sterilised with a 0.22μm filter (Nalgene). 100μl aliquots were stored at -20°C.

Poly-DL-ornithine: 0.5 mg/ml poly-DL-ornithine (Sigma) was prepared in 0.15M borate buffer (4.6g boric acid (BDH) in 500ml de-ionised, distilled water, pH 8.4) and filter sterilised using a 0.22μm bell filter (Gelman Sc).

Laminin: Murine laminin (Sigma) was thawed at 4°C and diluted in CMF-HBSS to give a final concentration of 20μg/ml.

L-15: A 1l unit of L-15 powder (GibcoBRL) was dissolved in 1l of de-ionised, distilled water containing 100mg streptomycin (Sigma) and 60mg penicillin (Sigma). The pH was adjusted to 7.3 and the solution sterilised with a 0.22μm bell filter.

Hams' F-12: A1l unit of F-12 powder (GibcoBRL) was dissolved in 1l of de-ionised, distilled water containing 100mg streptomycin (Sigma) and 60 mg penicillin (Sigma). The pH was adjusted to 7.3 and the solution sterilised with a 0.22μm bell filter. To prepare serum-supplemented medium 50ml of heat-inactivated horse serum (GibcoBRL) was added to 450ml 1x F-12.

Hams' F-14: 10x stock: A 5l unit of F-14 powder (Imperial labatories) was dissolved in 500ml of de-ionised, distilled water containing 500mg streptomycin (Sigma) and 300mg penicillin (Sigma). The 10x stock was stored in 50ml aliquots at -20°C.

1x solution: a 50ml aliquot of 10x F-14 was diluted to 500ml with de-ionised, distilled water containing 1g $NaHCO_3$ (BDH). The pH was adjusted to pH7 by bubbling through CO_2, after which the solution was sterilised with a 0.22μm bell filter.

SATO supplement: A stock solution was prepared containing the following reagents: 100ml Albumax-1 (GibcoBRL), 100ml ddH_2O, 160mg putrescine, 1ml progesterone (0.625mg/ml in ethanol, Sigma), 10ml L-thyroxine (0.4mg/ml in ethanol, Sigma). This stock solution was stored in 5.55ml aliquots at -20°C. To prepare the culture medium 5.55ml of SATO stock was diluted with 2.5ml 200mM L-glutamine (GibcoBRL) and 250ml 1x F-14.

Neurotrophic Factors: All neurotrophic factors were kept in stock aliquots of 10μg/ml in phophate-buffered saline (PBS, GibcoBRL) plus 10% HIHS, at 4°C, -20°C or -70°C as per manufacturers instructions. BDNF and NGF originated from..., while CNTF and LIF were from Chemicon.

Genotyping: Reaction mixes and cycling parameters

All genotyping reactions were performed using Hotstart Taq DNA polymerase and supplied buffers (Biogene, UK)

	IKKA	*p65*
10x buffer	2μl	2μl
$MgCl_2$	5μl	1μl
Mr solution	3.8μl	-
5mM dNTP	1μl	1μl
Primers	1μl or each primer	1μl or each primer
Taq DNA polymerase	0.15μl	0.15μl
dH_2O	6.5	14.25
Genomic DNA	1μl	1μl
Annealing temperature	55	57
Number of cycles	35	32

Competitive RT-PCR

All reactions were carried out using Superscript reverse transcriptase (Sigma) and Taq DNA polymerase (Helena Biosciences).

Reverse Transcription:

8µl 5x buffer

4µl DTT

4µl random hexamers

4µl dNTPs

1µl reverse transcriptase

5µl total RNA or sample

1µl Reg-2 competitor RNA (concentration calibrated for each set of samples)

1µl GAPDH competitor RNA (concentration calibrated for each set of samples)

12µl dH_2O

PCR: The sequences of the Reg-2 primers were

5'-GGACACCTCGTATCTGTGCTC-3' and

5'-TCCAAGTGTATTGGTAGCTGTTTC-3' and the reaction was carried out at an annealing temperature of 60°C for 35 cycles.

4µl 10x buffer

4µl primers (stock contains 1µg/µl of each primer)

1µl dNTP

0.125µl Taq DNA polymerase

5µl RT reaction

25.875µl dH_2O

Semi-quantitative RT-PCR: Reaction mix and cycling parameters

All reactions were carried out using Superscript reverse transcriptase (Sigma) and Taq DNA polymerase (Helena Biosciences).

Reverse Transcription:

8µl 5x buffer

4µl DTT

4µl random hexamers

4µl dNTPs

1µl reverse transcriptase

5µl total RNA or sample

14µl dH_2O

PCR:

2.5µl 10x buffer

1µl specific primers (stock contains 1µg/µl of each primer)

1µl GAPDH primers (stock contains 0.1µg/µl of each primer)

1µl dNTP

0.2µl Taq DNA polymerase

5µl RT reaction

14.3µl dH_2O

An annealing temperature of 56°C and 35 cycles were used.

The primer sequences were:

GAPDH:	5'-CTTCATTGACCTCAACTACATG-3'
	5'-GGCATGGACTGTGGTCATGA -3'
Reg-I:	5'-GCTGAAGAAGACCTGCCATC-3'
	5'-GTCGAGAAACAGAGACCCACT-3'
Reg-II:	5'-ATTGAAGACCGTTTGACCTG-3'
	5'-AGTCAGCGATACACAATA-3'
Reg-IIIα	5'-TGTCCTCCTTGGTGAACG-3'

	5'-CTTGAACTTGCAGACAAATG-3'
Reg-IIIβ	5'-GCCTGATGCTCTTATCTCAGG-3'
	5'-ACGGTCTAAGGCAGTAGATGG-3'
Reg-IIIγ	5'-GCTCCTATTGCTATGCCTTG-3'
	5'-AAGGATTCGTCTCCCAGTTG-3'
Reg-IIIδ	5'-AACTGTCTTCTCCACGCATC'-3'
	5'-CTCCACTTCCATCCATTTTC-3'